REMARKS

Claims 1-8 are pending in this application. Pending completion of a personal interview with the Examiner, Applicant has not canceled, added, amended claims in this application.

Applicants take this opportunity to thank the Examiner for reconsideration and withdrawal of the objection to the Specification and the rejection of claims 1, 2, and 5-8 under 35 U.S.C. §112, second paragraph.

1. Rejection of Claims 1-8 under 35 U.S.C. §112, first paragraph

The Office Action rejects claims 1-8 under 35 U.S.C. §112, first paragraph, for the following reasons:

> "[T]he specification, while being enabling for a method of treating cell lines established from samples of cystic fibrosis patient resulting from an abnormal expression of genes caused by aberrant splicing in cells, comprising administering to the cells, an naturally occurring alternative splicing factor (ASF) by transfected the cells with expression vector to produce the ASF, whereby the abnormal expression shifts towards normal expression of the gene, does not reasonably provide enablement for a method of treating individual suffering from a disease resulting from an abnormal expression of genes caused by aberrant splicing in cells, wherein the disease and the abnormal genes are not defined, comprising administering to the cells or to tissue or organs of the individual comprising the cells, an ASF, whereby the abnormal expression shifts towards normal expression of the gene. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims."

Specifically, the Examiner cites a purported lack of support for use of *in vitro* data to support *in vivo* usefulness, failure to satisfy a purported requirement to show routes of administration and dosage information in the Specification, and a purported lack of support for application of the cystic fibrosis data to broad, generic claims. The Examiner also notes that the Declarations filed on February 4, 2003 "are not found fully persuasive" for the same reasons.

Applicant respectfully traverses this rejection. Applicant furnishes four literature references which support the proposition that, to the ordinarily skilled artisan, the *in vitro* data readily support *in vivo* treatment, and that the claimed generic inventive subject matter, use of an ASF for treating an aberrant splicing disorder, finds broad support in the general knowledge which has developed in the art subsequent to the filing of this application.

Exhibit A: Jan-Gowth Chang, Hsiu-Mei Hsieh-Li, Yuh-Jyh Jong, Nancy M. Wang, Chang-Hai Tsai, and Hung Li, *Treatment of spinal muscular atrophy by sodium butyrate*, PNAS, 98:9808-9813 (2001). This study shows that sodium butyrate treatment of human SMA lymphoid cell lines increased the expression of full length, exon 7-containing SMN protein from the *SMN2* gene, by a mechanism which involves a change in the *SMN2* RNA splicing pattern. After sodium butyrate stimulation, both *in vitro* and *in vivo*, the transcription

pattern of *SMN2* changed to an *SMN1*-like transcription pattern, which was nearly identical to the SMN pattern in healthy individuals. *In vivo*, sodium butyrate treatment of SMA-like mice, by oral administration, resulted in increased expression of SMN protein in motor neurons of the spinal cord and resulted in significant improvement of SMA clinical symptoms. SMA symptoms were ameliorated for all three tested types of SMA-like mice. The authors conclude that these results suggest that sodium butyrate is an effective ASF for the treatment of human SMA patients.

Exhibit B: H. Zhao and G. A. Grabowski, *Gaucher disease: perspectives on a prototype lysosomal disease*, in *Human Genome and Diseases: Review*, Cell. Mol. Life Sci. 59:694-707 (2002). Gaucher disease is transmitted as an autosomal recessive trait. The 7.5-kb, 11 exon, gene encodes acid β-glucosidase (GCase, glucocerebrosidase, N-acyl-sphingosyl-β-D-glucose; glucohydrolase, EC 3.21.45). The GCase pseudogene is highly homologous to the normal GCase gene (96% identity). The GCase pseudogene is transcribed, but no functional protein results because of numerous mutations that generate stop codons. The major differences between the functional and pseudogenes are the presence of several splicing disorders, including small deletions in introns 2, 4, 6 and 7, numerous exonic missense mutations, and a 55-bp deletion in exon 9 in the pseudogene. The paper describes the "outstanding

accomplishment" of discovery of an effective and safe enzyme therapy for Gaucher disease, which has been reviewed extensively. The readers are referred to these references for details. Briefly, after a decade of enzyme therapy, the population of Gaucher patients have had major improvements in health and reversal of many aspects of their pathology. The article also discusses the "recent successes in gene therapy for immunodeficiency diseases" as an indication that progress is being made in this therapeutic strategy.

Exhibit C: David A. Buchner, Michelle Trudeau, Miriam H. Meisler, *SCNM1, a Putative RNA Splicing Factor That Modifies Disease Severity in Mice*, Science, 3011:967-969 (2003). This paper discusses the investigation into whether the mutant allele of gene *MGC3180/SCNM1* accounts for the disease susceptibility of mouse strain C57BL/6J to neurological disease and death. The authors tested the ability of the wild-type allele to rescue the lethal phenotype using a bacterial artificial chromosome. Purified BAC DNA was microinjected *in vivo* into fertilized eggs. A first generation transgenic founder was crossed to a congenic line, and the resulting transgenic offspring were backcrossed to the congenic line. The second generation offspring had a typical resistant phenotype demonstrating rescue by the BAC transgene. The abundance of the wild-type *MGC3180/SCNM1* transcript in brain RNA was 50% that

of the endogenous transcript. The level of correctly spliced transcript in rescued transgenic mice was comparable to the level previously measured in mice with the wild-type (resistance) allele, demonstrating transgenic rescue of the splicing defect.

Exhibit D: Joseph H. Nadeau, Modifying the Message, Science, 301:927-928 (2003). In a review of the Buchner, et al. Article (Exhibit C), the author discusses a rapidly growing body of evidence showing that various aspects of RNA biology, including splicing, are common targets of phenotypic modifiers, Applicant's work published in Trends Genet., 18:123 (2002) and previously provided to the Examiner. Mutations in variant motifs that include splice enhancers and splice silencers produce a mixture of normal and truncated transcripts and thus tend to have more modest and more variable effects. Again citing Applicants work, the author states that "modifier genes can act on the latter group by modulating the ratio of normal to abnormal transcripts. There is potential to design drugs that target modifier proteins and thus modulate the level of normally spliced transcripts."

Based on these literature references, it is apparent that Applicant's experimental and prophetic examples have been borne out, and that the ordinarily skilled artisan has been able to use methods disclosed by Applicant for using an ASF in the *in vivo* treatment of aberrant splicing disorders.

Finally, Applicant traverses this rejection because the Examiner's purported requirement of route of administration and dosage information in the Specification of this application misstates both the current state of the art in the field, and the applicable law, in asserting that a mere need for further experimentation is sufficient to support the rejection; the requirement is that additional experimentation be "undue." No more than routine experimentation, the methods of which are well known to those of ordinary skill in the art, is required to optimize dosage and route of administration.

Accordingly, Applicants respectfully request the Examiner to reconsider and withdraw the rejection of claims 1-8.

CONCLUSION

Based upon the above remarks, the presently claimed subject matter is believed to be novel and patentably distinguishable over the prior art of record. The Examiner is therefore respectfully requested to reconsider and withdraw the rejections of remaining claims 1-8 and allow all pending claims presented herein for reconsideration. Favorable action with an early allowance of the claims pending in this application is earnestly solicited.

The Examiner is welcomed to telephone the undersigned attorney if she has any questions or comments.

Respectfully submitted,

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Exhibit A

Treatment of spinal muscular atrophy by sodium butyrate

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Edited by Yuet Wai Kan, University of California, San Francisco, CA, and approved June 18, 2001 (received for review March 2, 2001)

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of the anterior horn cells of the spinal cord, leading to muscular paralysis with muscular atrophy. No effective treatment of this disorder is presently available. Studies of the correlation between disease severity and the amount of survival motor neuron (SMN) protein have shown an inverse relationship. We report that sodium butyrate effectively increases the amount of exon 7-containing SMN protein in SMA lymphoid cell lines by changing the alternative splicing pattern of exon 7 in the *SMN2* gene. *In vivo*, sodium butyrate treatment of SMA-like mice resulted in increased expression of SMN protein in motor neurons of the spinal cord and resulted in significant improvement of SMA clinical symptoms. Oral administration of sodium butyrate to intercrosses of heterozygous pregnant knockout-transgenic SMA-like mice decreased the birth rate of severe types of SMA-like mice, and SMA symptoms were ameliorated for all three types of SMA-like mice. These results suggest that sodium butyrate may be an effective drug for the treatment of human SMA patients.

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of anterior horn cells of the spinal cord, leading to muscular paralysis with muscular atrophy. Clinical diagnosis of SMA is based on findings of progressive symmetric weakness and atrophy of the proximal muscles. Affected individuals usually are classified into three groups according to the age of onset and progression of the disease. Children with type I SMA are most severely affected and usually have SMA symptoms before the age of 6 months and rarely live beyond 2 years. Type II and type III SMA are milder forms and the age of onset of symptoms varies between 6 months and 17 years. SMA is one of the most common fatal autosomal recessive diseases in children with a carrier rate of 1–2% in the general population and an incidence of 1 in 10,000 newborns (1). No specific treatment is currently available for SMA patients.

Two survival motor neuron (SMN) genes (*SMN*) are typically present on 5q13: *SMN1* (also known as *SMNT*, *SMNtel*) and *SMN2* (also known as *SMNc*, *SMNcen*). Loss-of-function mutations of both copies of the telomeric gene, *SMN1*, are correlated with the development of SMA (2–5). The nearly identical centromeric gene, *SMN2*, appears to modify disease severity in a dose-dependent manner, as SMN protein levels from this gene are correlated with disease severity (6, 7). However, the expressed amount of intact SMN protein from *SMN2* does not provide adequate protection from SMA (8).

Although *SMN1* and *SMN2* encode identical proteins, all three forms of proximal SMA are caused by mutation in the *SMN1* gene, but not in the *SMN2* gene (2–5). The differences between these highly homologous genes are in their RNA expression patterns (9–12). Most *SMN2* transcripts lack exons 3, 5, or most frequently, 7, with only a small amount of full-length mRNA generated. On the other hand, the *SMN1* gene expresses mostly a full-length mRNA, and only a small fraction of its transcripts are spliced to remove exons 3, 5, or 7 (11, 12). Recent studies also have shown that an AG-rich exonic splice enhancer in the center of *SMN* exon 7 is required for constitutive inclusion of exon 7 (13). These findings also imply that the low levels of full-length SMN protein produced by *SMN2* are insufficient to protect against disease development (6, 7). Clearly, the total amount of full-length oligomerization-competent SMN protein is a critical SMA determinant, and the amount of SMN protein correlates with the severity of pathologies (14). In addition, there is a strong correlation between the *SMN2* copy number and phenotype in human SMA and SMA-like mice (5–7, 15, 16).

We recently developed a SMA mouse model that genotypically and phenotypically mimics human SMA (15). The severity of pathology in the knockout-transgenic mice is correlated with the amount of intact SMN protein. The difference between *SMN1* and *SMN2* gene expression is the number of full-length transcripts and the amount of SMN protein, and all 5q-linked SMA patients have at least a single intact copy of *SMN2*. Drugs that modify the pattern of *SMN2* transcript in SMA patients to increase full-length *SMN* mRNA expression and the amount of SMN protein may have a therapeutic effect on SMA patients. As a step toward designing a therapeutic protocol for SMA patients, we used Epstein–Barr virus-transformed lymphoid cell lines from SMA patients to screen a series of drugs for their possible effect on the expression of the *SMN2* gene. One drug that was found to be effective was then used to treat our SMA-like mice to determine its potential for the treatment of human SMA.

SR proteins (Ser-Arg proteins) constitute a family of pre-mRNA splicing factors that are highly conserved throughout the metazoa (17, 18). These proteins have multiple functions in splicing. Biochemical experiments have provided strong evidence that SR proteins play essential roles in general, or constitutive, splicing. They seem to be equally important in splicing regulation, through their ability to modulate selection of alternative splicing sites in a concentration-dependent manner, which contributes to activation (and repression) of splicing through interaction with elements in the pre-mRNA known as splicing enhancers (or silencers) (19–21). Recently, Lorson and Androphy (13) demonstrated that an AG-rich exonic splice enhancer in the center of *SMN* exon 7 is required for inclusion of exon 7 and further demonstrated that Htra2-β1, an SR-like splicing factor, promoted the inclusion of *SMN* exon 7, stimulating full-length *SMN2* expression. Htra2-β1 specifically functioned through and bound to an AG-rich exonic splicing enhancer in *SMN* exon 7 (22). In the present study, we have explored the relationship between the drug's effect and SR protein.

Materials and Methods

Cell Culture. We established Epstein–Barr virus-transformed lymphoid cell lines from different SMA-type patients (five cases

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Abbreviations: SMA, spinal muscular atrophy; SMN, survival motor neuron; RT-PCR, reverse transcriptase–PCR.

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each for types I, II, and III) with deleted *SMN1* genes by using the following procedures (4). Lymphocytes were collected from whole blood of patients by Ficoll hypaque separation. The buffy coat was collected and washed twice with 5 ml PBS. The pellet was resuspended in 5 ml RPMI medium containing 0.5 ml Epstein–Barr virus, 50 μl phytohemagglutinin (0.5 mg/ml), and 50 μl cyclosporine (0.2 mg/ml). Cells were incubated at 37°C with 5% CO_2 until they became viable.

Mice. Five independent human *SMN2* gene transgenic mice were generated and crossed with mice heterozygous for the *Smn* locus knockout. Transgenic mice that were also homozygous for the knockout alleles ($Smn^{-/-}$ *SMN2*) were then generated by crossing with the above mice. These knockout-transgenic mice developed progressive motor-neuron disease similar to that in human SMA patients. The SMA-like mice were classified into three groups based on their phenotypes, which were judged by three authors (J.-G.C., H.-M.H.-L., and H.L.). Mice with the most severe pathological form (type 1) did not develop furry hair and died before postnatal day 10; mice with intermediate severity (type 2) showed poor activity and variable symptoms and died at ≈2–4 weeks; the type 3 mice survived and bred normally, but had short and enlarged tails (15). SMA-like mice (nonpregnant and pregnant) were supplied with sterile water ad libitum and rodent pellets. The sodium butyrate-treated group received sodium butyrate at a concentration of 0.8 mg/ml or 8 mg/ml in distilled water (with no other substances added), beginning immediately after diagnosis or after 15 days of gestation in SMA-like pregnant mice. Both groups consumed ≈5–10 ml per day per mouse. After 1–12 weeks of treatment, the mice were killed, and their organs or tissues were quickly removed and frozen in liquid nitrogen.

Reverse Transcriptase–PCR (RT-PCR) Analysis. RT from total RNA was performed by using a random primer 5′-TN_{10}-3′ and Moloney murine leukemia virus RT. PCR was then used to amplify the single-stranded cDNA by using one or three pairs of primers covering the entire *SMN* coding region. The first primer pair used to amplify the fragment from the 5′ untranslated region to exon 4 was: forward primer, P1, 5′-CGCTGCGCATCCGCGGGTTTGCTATGGC-3′ and reverse primer, P2, 5′-TCCCAGTCTTGGCCCTGGCAT-3′. The second primer pair used to amplify exons 4–6 was: forward primer, P3, 5′-AACATCAAGCCCAAATCTGC-3′ and reverse primer, P4, 5′-GCCAGTATGATAGCCACTCATGTACCATG-3′. The third primer pair amplified from exon 6 to exon 8 was: forward primer, P5, 5′-CTCCCATATGTCCAGATTC-TCTTGATGATGC-3′ and reverse primer, P6, 5′-ACTGCCTCACCACCGTGCTGG-3′. P1 and P6 were used to amplify the full-length *SMN* cDNA. The PCRs were performed as described (15).

Subcellular Fractionation. Fresh frozen spinal cord, brain, and skeletal muscle samples (500 mg) from different types of SMA mice were fractionated as described (15). Tissues were homogenized with a tight-fitting glass pestle in ice-cold buffer A (10 mM Hepes, pH 7.9/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/1 mM DTT/0.5 mM PMSF/2 μg/ml leupeptin/2 μg/ml pepstatin) with 0.5% Nonidet P-40 and kept on ice for 15 min. The nuclei were pelleted by centrifugation at 800 *g* for 3 min. The nuclear pellet was resuspended by trituration in 100 μl of buffer B (20 mM Hepes, pH 7.9/0.4 M NaCl/1 mM EDTA/1 mM EGTA/1 mM DTT/1 mM PMSF/2 μg/ml leupeptin/2 μg/ml pepstatin) and kept on ice for 15 min followed by centrifugation at 15,000 *g* for 10 min at 4°C. The supernatant (soluble nuclear extract) was removed, and the insoluble nuclear pellet was further sonicated in sonication buffer (100 mM Tris·HCl, pH 7.4/1% SDS/5 mM EDTA/1 mM DTT/1 mM PMSF/2 μg/ml leupeptin/2 μg/ml pepstatin).

Western Blot Analysis and Histopathological Analysis. Synthetic peptides containing part of human SMN exon 7 (amino acids 279–288) and exon 2 (amino acids 72–84) were used to immunize rabbits and to purify specific antibodies (H2 and H7) from rabbit crude sera with an EAH-Sepharose 4B column (Amersham Pharmacia) according to the manufacturer's instructions. Two mouse anti-SR protein antibodies (anti-SRp20 and 16H3), purchased from Zymed, were used to detect the human SR proteins. Protein samples were loaded on a 5% polyacrylamide stacking gel above a 12% separating gel, and the gel was run with a discontinuous buffer using Laemmli's method. After electrophoresis, proteins were transferred electrophoretically to poly(vinylidene difluoride) membranes (Millipore). After the transfer, the membranes were blocked in TBST (50 mM Tris·HCl, pH 7.5/150 mM NaCl/0.05% Tween-20) containing 4% BSA for 2 h at room temperature. Blots were incubated with adequate dilution of anti-SMN exon 2 (H2), anti-SMN exon 7 (H7), or anti-SR protein antibodies in TBST for 2 h at room temperature. The blots were washed for three 20-min periods in TBST and then incubated with a 1:32,000 dilution of an anti-rabbit IgG alkaline phosphatase conjugate (Sigma) in TBST for 1 h at room temperature. The reaction was detected by adding 1.5% 5-bromo-4-chloro-3-indoyl phosphate and 3% nitro blue tetrazolium in a developing buffer (100 mM NaCl/5 mM $MgCl_2$/100 mM Tris·HCl, pH 9.5). Histopathological analysis and immunohistochemical staining were performed as described (15).

Statistics. The intensity of the RT-PCR products containing exon 7, lacking exon 7, or SMN and tubulin in Western blot were analyzed by the Collage Image Analysis System to calculate the ratio of these products (12). Results from multiple experiments are expressed as mean ± standard error. Survival data of treated and untreated mice are presented as a Kaplan–Meier plot using the log rank test. A standard χ^2 test was used to assess differences in the frequency of mild or severe phenotype in the SMA-like mice born from treated and untreated mothers, which analyzed the percentage of type 1 (or 2 + 3) newborn mice as a fraction of the total number of pups. Differences with a P value of <0.05 were considered statistically significant.

Results

Sodium Butyrate Changes the Processing of *SMN2* Gene Transcripts. Epstein–Barr virus-transformed lymphoid cell lines from all three types of SMA patients were established and used for drug screening. Several drugs were tested to investigate their potential effect on the expression of the *SMN2* gene by using RT-PCR. Among them, sodium butyrate was able to change the expression pattern of the *SMN2* gene. The amount of exon 7-containing *SMN* mRNAs increased in lymphoid cells cultured with 5 ng/ml to 500 μg/ml of sodium butyrate (Fig. 1*a*). The maximal effect was found after 4 h of stimulation (Fig. 1*b*). Sodium butyrate-treated lymphoid cells from all types of SMA patients showed an increased number of full-length *SMN* transcripts (Fig. 1c). To understand the mechanism involved in this change in full-length *SMN* transcript levels, separate RT-PCRs were used to examine the patterns of alternative splicing in exons 3, 5, and 7. We found that the alternative splicing pattern of exons 3 and 5 was unchanged after sodium butyrate stimulation (Fig. 1 *d* and *e*), but that the alternative splicing pattern of exon 7 of the *SMN2* gene changed to the *SMN1* pattern (Fig. 1*f*). Therefore, addition of sodium butyrate in the culture resulted in an increased number of full-length *SMN* mRNA transcripts.

Sodium Butyrate Increases Exon 7-Containing SMN Protein in SMA Lymphoid Cells. To determine whether sodium butyrate-induced expression pattern changes of *SMN2* resulted in an increased amount of exon 7-containing SMN protein, we used different concentrations of sodium butyrate to treat the lymphoid cell

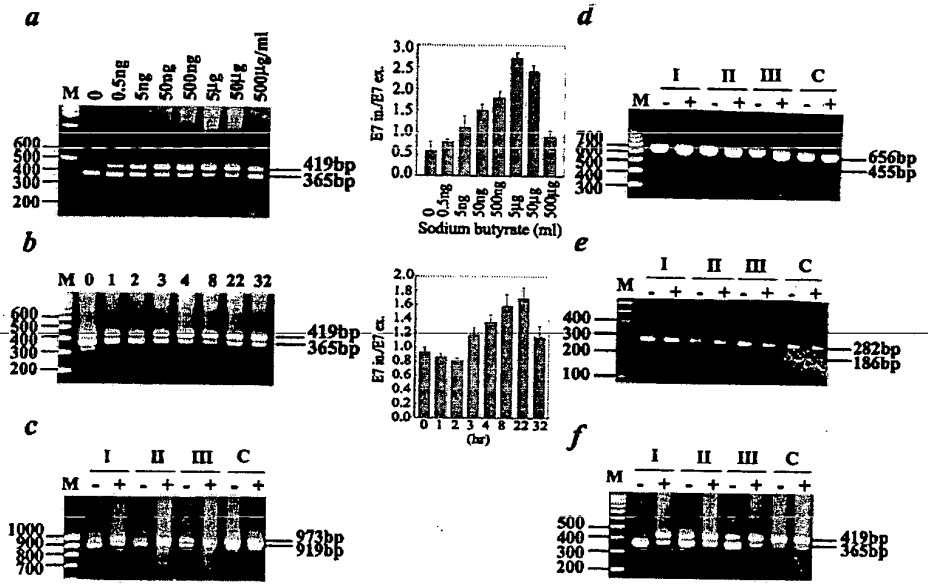

Fig. 1. Effects of sodium butyrate on the expression of the human *SMN2* gene in lymphoid cell lines of SMA patients with *SMN1* deletion. (*a*) RT-PCR analyses of exons 6–8 of the *SMN2* gene showed that the exon 7-containing transcript was increased with 5 ng/ml to 500 μg/ml sodium butyrate treatment. Quantitative analysis of the exon 7-containing transcript is shown on the right, in which the ratio of exon 7 inclusion to exon 7 exclusion is indicated (mean ± SD; $n = 3$). M: 100 bp-ladder marker. E7 in.: exon 7 inclusion; E7 ex.: exon 7 exclusion. (*b*) The SMA lymphoid cell lines were treated with sodium butyrate for 1, 2, 3, 4, 8, 22, and 32 h. RT-PCR analyses of exons 6–8 of the *SMN2* gene showed that the exon 7-containing transcript was increased after 4-h treatment with 500 ng/ml sodium butyrate. A quantitative analysis of the exon 7-containing transcript is shown on the right (mean ± SD; $n = 3$). (*c*) RT-PCR amplification of whole cDNAs (exons 1–8) of *SMN2* genes from different types of SMA lymphoid cell lines showed that the full-length transcript of the *SMN2* gene is very similar to the transcript of the *SMN1* gene after sodium butyrate treatment. (I, II, and III represent different types of SMA lymphoid cell lines; −, untreated; +, treated; C, normal). (*d–f*) RT-PCR analyses of exons 1–4 (*d*), 4–6 (*e*), and 6–8 (*f*) for *SMN2* gene expression showed that only the transcript pattern of exon 7 was changed due to alternative splicing. There was no change for exons 3 and 5.

lines (three cases each for types I, II, and III) established from different types of SMA patients. In both cytosolic and nuclear fractions, Western blot analysis indicated that sodium butyrate also increased the intact SMN protein after 4-h stimulation with 0.5 ng/ml to 500 μg/ml of sodium butyrate (Fig. 2). However, a decreasing effect was found in the cytosolic fraction when more than 5 μg/ml sodium butyrate was used.

Sodium Butyrate Increases Specific SR Proteins in SMA Lymphoid Cell Lines. SR proteins are known to play an important role in the [illegible] studies have identified a splicing enhancer element in exon 7 of the *SMN* gene (10, 13). To investigate sodium butyrate-induced expression pattern changes of *SMN2* involving the SR protein, we used different antibodies for SR proteins to detect SR protein expression patterns after sodium butyrate treatment. The results showed that two SR proteins of about 27 kDa were induced after treatment, which were detected by using mouse anti-SR protein 16H3 antibody. However, no difference was found by using the mouse anti-SRp20 antibody (Fig. 3*a*). These induced SR protein reactions were blocked by either a specific mitogen-activated protein kinase inhibitor (PD98059) or an inhibitor of protein phosphatases (okadaic acid) (Fig. 3*b*). All lymphoid cell lines (three cases each for types I, II, and III), which were established from different types of SMA patients, showed similar results.

Treatment of Types 2 and 3 SMA-Like Mice with Sodium Butyrate. To investigate whether the *in vitro* effects of sodium butyrate on lymphoid cell lines also occur in SMA-like mice *in vivo*, we used sodium butyrate to treat types 2 and 3 SMA-like mice (15 mice each). Sodium butyrate was administered to SMA-like mice via a 0.8 mg/ml or 8 mg/ml solution available ad libitum in their drinking water for 1–12 weeks. The amount of sodium butyrate consumed by SMA-like mice was estimated to be ≈4–80 mg/day. The sodium butyrate-treated type 2 SMA-like mice survived 4–5 days longer than the untreated ones (Fig. 4). Some of the treated type 2 mice ultimately died from infection caused by traumatic injury of the paralytic hindlimbs.

Our previous study showed that tails of untreated types 2 and 3 SMA-like mice had decreased diameters of muscle fibers, atrophy of muscle bundles, group atrophy, and subcutaneous edema (15). In the present study, after sodium butyrate treatment, the tails of types 2 and 3 SMA-like mice showed nearly normal muscle patterns. Grossly, the tails of treated mice were slightly shorter than normal, and treated mice rarely developed chronic necrosis from the tip of the tail toward the root (2% for the treated group vs. 50% for the untreated group). Histopathologically, the tails of treated mice had few atrophied muscle bundles, and group atrophy and subcutaneous edema were rarely present (Fig. 5). Western blot analysis showed that the exon 7-containing SMN protein level was elevated in different tissues, including motor neurons of the spinal cord (Fig. 6 *a* and *b*). Immunohistochemical studies showed that both exon 2-containing (Fig. 6 *c* and *d*) and exon 7-containing (Fig. 6 *e* and *f*) proteins were increased, which may have resulted from an increase in the total amount of intact SMN protein. Because the severity of the pathological changes in SMA patients and SMA-like mice is correlated with the amount of intact SMN protein present in the spinal cord (6, 7, 15, 16), the effect of oral sodium butyrate,

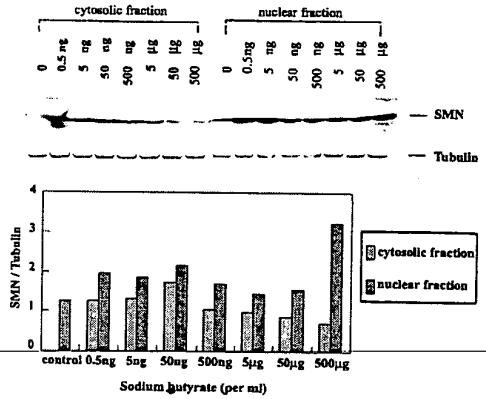

Fig. 2. Effects of sodium butyrate on the expression of the human SMN protein in lymphoid cell line of a representative SMA type I patient. (*Upper*) Western blot analysis showed a gradual increase in exon 7-containing SMN protein expression in nuclear fraction after 0.5 ng/ml to 500 μg/ml sodium butyrate treatment. The amount in the cytosolic fraction decreased with increasing doses (5, 50, and 500 μg) of sodium butyrate. (*Lower*) Quantitative analysis of SMN and tubulin ratios are shown. The control represents an untreated SMA control.

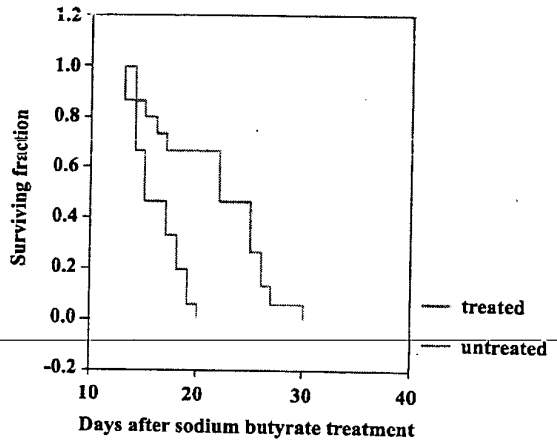

Fig. 4. Survival time of type 2 SMA-like mice after sodium butyrate treatment shown in a Kaplan–Meier survival curve. Sodium butyrate was added to the drinking water after diagnosis of type 2 SMA-like mice that showed poor activity after postnatal day 10; 15 mice were left untreated (red), and 15 mice were treated with sodium butyrate (green) from diagnosis to death. The type 2 SMA-like mice treated with sodium butyrate lived significantly longer than those in the untreated SMA control group ($P = 0.0004$). Treated group: mean = 21.7 days, range = 14–30 days; untreated group: mean = 15.5 days, range = 13–20 days.

particularly in the spinal cord, may be of therapeutic value for SMA patients.

We also used 16 mg/day and 40 mg/day sodium butyrate solution for the treatment and found no definite toxicity at 16 mg/day sodium butyrate treatment, whereas mice that received 40 mg/day sodium butyrate treatment died because of dehydration.

Treatment of Intercross Heterozygous Knockout-Transgenic Mice after Pregnancy. Because the survival time of type 1 and some type 2 SMA mice is short, evaluation of the therapeutic effect of sodium butyrate is difficult. To overcome these problems, sodium butyrate (4–80 mg/day) was administered ad libitum in drinking water to pregnant $Smn^{+/-}SMN2$ intercrossed mice, which had previously produced offspring of different types of SMA progeny, especially the severe form (15). Sodium butyrate treatment began on the 15th day postcoitum to avoid a possible teratogenic effect. A total of 21 pups with type 1, 22 with type 2, and 48 with type 3 were born from the treated group; and 35 pups with type 1, 17 with type 2, and 38 with type 3 were born

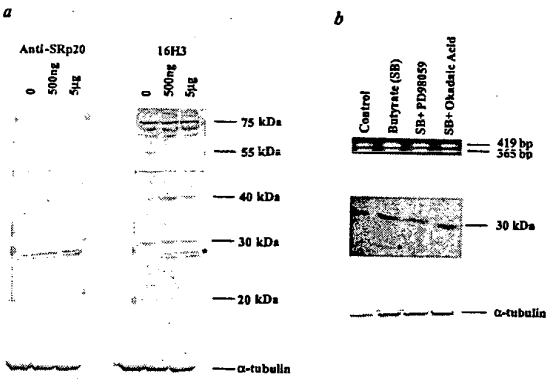

Fig. 3. Effects of sodium butyrate on the expression of human SR proteins in the lymphoid cell line of a representative SMA type I patient. (*a*) Western blot analysis showed two SR proteins (•) were induced after 500 ng and 5 μg sodium butyrate stimulation, which were detected by mouse anti-SR protein mAb (6H3), but no difference was found by using mouse anti-SRp20 mAb. (*b*) The induced SR proteins (•) disappeared after adding mitogen-activated protein kinase inhibitor PD98059 or phosphate inhibitor (okadaic acid), and the expression pattern of *SMN2* also changed to untreated status. The control represents an untreated SMA control.

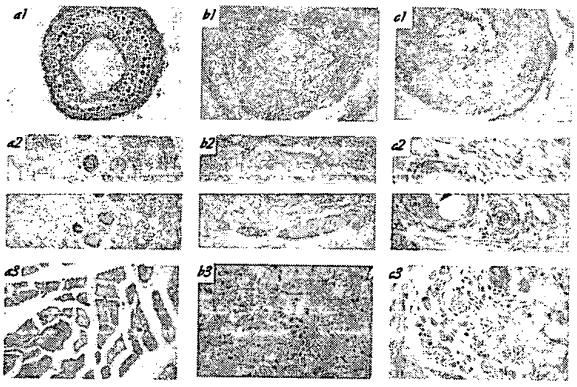

Fig. 5. Histological analysis of a type 3 SMA-like mouse after sodium butyrate treatment. (*a1–a3*) Normal control. (*b1–b3*) Type 3 SMA-like representative mouse after sodium butyrate treatment. (*c1–c3*) Type 3 SMA-like representative mouse with no treatment. Cross sections of tails were stained with hematoxylin and eosin (×40 for *a1*, *b1*, and *c1*; ×100 for *a2* and *b2*; ×400 for *c2*, *a3*, *b3*, and *c3*). The tails of sodium butyrate-treated mice had a few regions of muscular bundle atrophy but no subcutaneous edema (*b1–b3*). The tails of untreated mice had severe muscular bundle atrophy (*c1* and *c3*), thrombus in the vessel walls due to muscular atrophy, resulting in venous blood stasis (*c2*, arrow), and mild subcutaneous edema (*c1*) compared with normal controls (*a1–a3*).

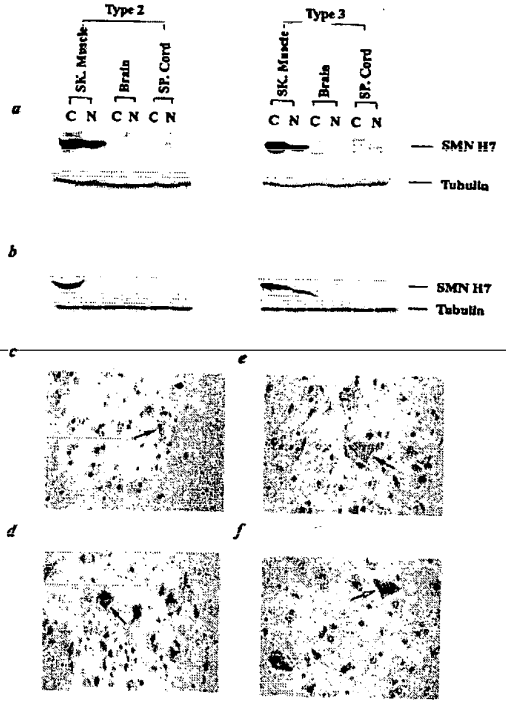

Fig. 6. Expression of human exon 7-containing SMN protein in types 2 and 3 SMA mice after sodium butyrate treatment. (*a*) Western blot analysis of exon 7-containing SMN protein (detected by SMN H7 antibody) in cytosolic (C) and nuclear (N) fractions from different tissues. The exon 7-containing SMN protein in treated mice was increased in skeletal muscle (SK. Muscle) and spinal cord (SP. Cord) after sodium butyrate treatment, especially in the nuclear fraction, compared with untreated types 2 and 3 mice. (*b*) Control incubation was performed with an anti-α-tubulin antibody to determine the relative amount of SMN protein (Tubulin). (*c–f*) Immunohistochemical staining of anterior horn cells of the spinal cord showed an increase of SMN protein in motor neurons of type 2 SMA-like mice after sodium butyrate treatment. (*c*) Untreated (H2 antibody). (*d*) Treated (H2 antibody). (*e*) Untreated (H7 antibody). (*f*) Treated (H7 antibody). Arrow: motor neuron.

from the untreated group (Table 1). These results show that treatment with sodium butyrate from day 15 of pregnancy significantly ameliorated the clinical symptoms of the severe SMA phenotype, leading to milder types of SMA in offspring (Table 1). In addition, fewer SMA-like mice were born in the untreated group, which may have been due to some severe-type mice being aborted in the fetal stage or eaten by their mothers after birth, and thus remaining uncounted.

Table 1. Phenotype comparison and statistical analysis between pups from sodium butyrate-treated and nontreated mothers (control)

Mouse	Sodium butyrate	Control
Litters	32	46
Total pups	294	364
Type 1	21	35*
Type 2	22	17
Type 3	48	38

*, $P < 0.05$ for statistical analysis of type 1 and type 2 + 3 between treated and untreated groups.

Discussion

The amount of exon 7-containing SMN protein has been shown to be an inverse indicator of disease severity in SMA patients and mice (6, 7, 15, 16). Therefore, increasing the expression of intact SMN protein may have clinically therapeutic effects on SMA patients. In this study, we found that sodium butyrate treatment of human SMA lymphoid cell lines increased the expression of exon 7-containing SMN protein from the *SMN2* gene. The mechanism by which sodium butyrate affects SMN protein expression of the *SMN2* gene involves a change in its RNA splicing pattern of the gene. After sodium butyrate stimulation *in vitro* and *in vivo*, the transcription pattern of *SMN2* changed to an *SMN1*-like transcription pattern, which was nearly identical to the *SMN* pattern in healthy individuals. These findings may have important implications regarding the treatment of SMA patients.

Sodium butyrate has been shown to induce differentiation and apoptosis (23, 24). There is evidence that sodium butyrate may act at the transcription level by increasing the acetylation of histones, thereby releasing constraints on the DNA template and reactivating a number of genes (25, 26). Sodium butyrate also increases the expression of fetal-globin genes in adult baboons, humans, and other animals (27–29). *In utero* infusions of butyrate delay the developmental switch from γ- to β-globin gene expression in sheep fetuses (29). These effects of butyrate may occur through the inhibition of histone deacetylase (25, 26, 29, 30). In the case of *SMN*, sodium butyrate may acetylate nucleosomal DNA and release other factors that control alternating splicing of exon 7 of the *SMN2* gene.

We demonstrated that sodium butyrate induced two specific SR proteins involved in inclusion of exon 7 for full-length *SMN* expression of the *SMN2* gene. These reactions were blocked by either the mitogen-activated protein kinase inhibitor or a phosphatase inhibitor. Our results strongly support that SR proteins are involved in *SMN2* exon 7 inclusion after sodium butyrate treatment.

Approximately 15% of all mutations that cause genetic diseases result from the defective splicing of pre-mRNA (31). A number of these mutations do not alter consensus splice sites or generate missense or nonsense mutations, yet do affect splice site selection (32, 33). These mutations may cause skipping of exon(s) by disrupting the splicing enhancer(s). Our findings suggest that an approach similar to that used in our study may be effective in treating these kinds of genetic diseases as well.

Most SMA patients gradually develop clinical symptoms after birth. We previously demonstrated that the *SMN2* in SMA-like mice expressed only a decreased or nearly normal amount of intact SMN protein in most tissues, except in motor neurons (15). This is why SMA is a disease that directly affects only the motor neurons. The motor neuron-specific splicing factors regulating the inclusion/exclusion of exon 7 in the fetal stage, which are shut down in the spinal cord after birth, may account for the specific defect present in SMA. These factors also may play an important role in genotypic and phenotypic discrepancies. Sodium butyrate inhibits the deacetylation of these phenotype-related genes, modifying the clinical symptoms of SMA in a fashion similar to the mechanism involved in fetal hemoglobin gene expression (25, 26, 30). However, there is a major difference between modification of the γ-globin gene and the *SMN2* gene after butyrate reaction. The transcription of the *SMN2* gene is modified through the alternative splicing of exon 7 rather than directly through the inhibition of histone deacetylation. Gene modification after sodium butyrate treatment not only increased the transcription of *SMN2*, but also changed the splicing pattern of exon 7 of *SMN2* whereas the splicing pattern of exons 3 or 5 remained unchanged. This may be caused by the influence on

exon 7 inclusion of specific SR proteins that are induced by sodium butyrate treatment.

Sodium butyrate and related compounds have been used clinically to treat patients with sickle cell anemia and thalassemia for several years (34, 35). The pharmacokinetics and toxicities of sodium butyrate are well documented; its toxicity is low and has been well tolerated in both human and animal studies (34–37). Our findings suggest that sodium butyrate is an excellent candidate for the treatment of human SMA. In the present study, although sodium butyrate had a therapeutic effect on SMA symptoms, a number of severe types of SMA mice were born to sodium butyrate-treated pregnant mice and a few type 2 mice showed poor response after sodium butyrate treatment. This may have been due to incomplete treatment, or because the increase in the amount of intact SMN protein after treatment was unable to sufficiently compensate, to provide the minimal requirement for motor neuron survival. It is also possible that the timing of treatment was too late after day 15 of pregnancy.

In summary, SMA lymphoid cell lines and SMA-like mice were used to explore possible medication for the treatment of human SMA in this study. We found that sodium butyrate can effectively treat SMA-like mice by changing the expression pattern of *SMN2* and increasing the amount of full-length mRNA of *SMN2* both *in vitro* and *in vivo*. The methods developed in this study may be useful in screening other candidate drugs for SMA treatment. We also demonstrated that the mechanism of action of sodium butyrate involves a modification of the splicing of exon 7 of the *SMN2* gene under the regulation of SR proteins. This study shows that a deacetylase inhibitor can specifically modulate a disease-related defect gene to change its expression pattern, resulting in amelioration of the related symptoms. Our methods also may provide a useful approach for the treatment of other splicing defect-related diseases (31, 38).

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Exhibit B

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CMLS Cellular and Molecular Life Sciences

Human Genome and Diseases: Review

Gaucher disease: perspectives on a prototype lysosomal disease

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Abstract. Gaucher disease is an autosomal recessive trait and the most common lysosomal storage disease. The pathogenesis evolves from the diminished activity of the lysosomal hydrolase, acid β-glucosidase and the resultant accumulation of glucosylceramide within lysosomes. The pathogenic mechanisms are poorly understood. During the past 2 decades, progress has been made in understanding the biochemical basis and molecular biology of the disease, but more fundamental knowledge is required to relate these advances to the cell and whole body phenotypes. Despite this lack of understanding, enzyme replacement therapy has proved a successful and effective management for Gaucher disease. However, basic details of this therapeutic efficacy require elucidation. Here, we review the current state of the molecular pathogenesis and provide our perspective of some major issues for continued advances in this prototype lysosomal storage disease.

Key words. Glucosidase; glucocerebrosidase; glucocerebroside; glycosphingolipid; macrophage; chitotriosidase; cytokines; enzyme therapy; gene therapy.

Introduction

In 1882, Dr Phillipe Gaucher, a French dermatologist, first described a neurologically normal 32-year-old female with massive hepatosplenomegaly [1]. He thought her disease was an epitheloma of the spleen because of the presence of peculiar cells in that organ (fig. 1A). In 1901, Brill recognized the systematic and familial nature of the disease and coined the term 'Gaucher's disease'. In 1907 the biochemical nature of Gaucher disease was recognized and, later, Aghion [2] characterized the storage material as glucosylceramide (GC). Since that time, much of the phenotypic diversity of Gaucher disease has been delineated, particularly the recognition of neuronopathic variants and variation within the defined types [3–6].

The disease encompasses a heterogeneous group of disorders with highly variable phenotypes caused by the defective lysosomal hydrolysis of GCs and related glucosphingolipids. Brady and co-workers, and Patrick identified a glucocerebrosidase, a GC glucohydrolase, as the enzymatic defect in Gaucher disease [7,8]. Later, this enzyme was shown to be a β-glucosidase [9]. The complementary DNA (cDNA) and gene were characterized, and about 200 mutations at this locus on human chromosome 1q 21–23 have been found in Gaucher disease patients [3]. Although an uncommon metabolic disorder, Gaucher disease is the most common lysosomal storage disease, with an estimated birth frequency of 1/50,000 in the Caucasian population [10]. The disease is panethnic and has its highest prevalence in the Ashkenazi Jewish population. The past 2 decades have witnessed much progress in understanding of biochemical and molecular basis of the disease, and their association with clinical presentation. Major advances include the availability of safe and effective enzyme therapy as a prototype for other such intracellular protein deficiency diseases. This re-

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view will provide an overview of some of the outstanding issues in understanding Gaucher disease as a prototype for other monogenic diseases due to enzyme deficiencies. Detailed reviews of the clinical and biochemical aspects of this disease are available [3,11–16].

Clinical and pathologic features

Clinical presentation

Development of perspectives on the cellular, biochemical and molecular aspects of Gaucher disease requires some insight into the clinical and pathologic phenotypes. Gaucher disease is classically divided into three variants based on the absence or presence and progressivity of neuronopathic disease [3, 6]. Table 1 presents a current summary of Gaucher disease classification. However, extensive variability exists within each phenotype, and the spectrum of involvement is great within each type. All variants have differing degrees of enlargement of the liver and spleen, anemia, thrombocytopenia and skeletal disease. These can range from very severe to mild within each type, although the rate of progression generally is greater in younger patients. Also, the degrees of visceral organ involvement are not concordant in patients. For example, massive involvement of the liver and spleen is not necessarily accompanied by severe bony disease. The reverse also is true. In addition, this classification is not age dependent, but depends on the primary involvement of the CNS by Gaucher disease at any age.

Gaucher disease type 1 patients are free of primary CNS involvement. The variability of the phenotype of visceral manifestations ranges from severe fatal disease in the first 2 decades to essentially asymptomatic nonagenarians. Gaucher disease types 2 and 3 have primary CNS neuronopathic involvement. Types 2 and 3 represent a continuum of disease phenotypes that differ, primarily in their rates of CNS and visceral disease progression. This continuum encompasses phenotypes leading to death in utero, or in the first few days of life, to rapidly progressive CNS and visceral diseases that are fatal in the first years, to more slowly progressive (yet severe) CNS (with mild to severe visceral disease) deterioration over a period of 2–3 years to decades [17–21]. For the neuronopathic variants, the brainstem and cranial nerve nuclei are variably involved, but eye movement abnormalities generally are the first findings. Brain stem (bulbar) findings predominate in the type 2 variants, but oculomotor apraxia may be the only finding in the type 3 variants for many years. Clinically, the distinctions between types 2 (acute neuronopathic) and 3 (subacute neuronopathic) are useful and have therapeutic import. Biologically, the distinctions may relate more to rate of substrate accumulation than qualitative mechanistic differences.

Table 1. Gaucher disease: General Classification of Clinical Variants

Clinical features	Type 1	Type 2	Type 3
Onset	early childhood/ adulthood	infancy	early childhood/ adolescence
Hepatosplenomegaly	+→+++	+++	+→+++
Hypersplenism	+→+++	+	+→+++
Bone Crises/ Fractures	+→+++	–	++
Neurodegenerative Course	–	+++	+→+++
Survival	< 6–80 + years	< 2 year	1st – 4th decade
Ethnic Predilection	panethnic Ashkenazi Jewish	panethnic	panethnic Northern Sweden

The gaucher cell

The presence of Gaucher cells in various tissues is the hallmark of this disease (fig. 1A). The Gaucher cell results from the accumulation of excessive glucocerebrosides in lysosomal compartments of monocyte/macrophage derived cells. Histologically, Gaucher cells are enlarged macrophages (up to 100 μm) with cytoplasmic linear inclusions. Ultrastructurally, these inclusions are tubule-like structures that contain GC (fig. 1B), i.e., the stored GC and phospholipids (~90%) and about 0.3% protein [22]. Ultrastructural studies of monocytes from Gaucher patients also show smaller membrane-bound tubule-like structures similar to those in Gaucher cells. These results indicate that monocytic cells are precursors of tissue-bound Gaucher cells [23]. The relationship of the accumulation of these cells to the overt clinical findings is not clear. For example, in some Gaucher disease patients, the liver and spleen may exceed 25% of body weight (normal < 3% of BW), but GC does not account for this excess mass. Normal tissue components with fibrosis and parenchymal expansion also are found, and may be a reaction to the Gaucher cells or the by-products of tissue injury. Thus, growth of normal tissue and reaction to the Gaucher cells requires explanation. The evolution of differential gene expression and pathologic response in these involved tissue and from Gaucher cells remains a fertile area for research.

The relationships of Gaucher cells to cortical bone loss (osteopenia and osteoporosis) and bone marrow disease are even more obscure. Necrosis, fibrosis and Gaucher cell infiltration account for some of this pathology, but hyperemia and ischemia also may be critical. A recent study showed increased cathepsin K expression in Gaucher cells [24]. This protein has high-level expression in osteoclasts, a cell to which its expression is restricted. Cathepsin K has roles in bone resorption, modeling and turnover [25,26]. This protease and other cytokines overexpressed by Gaucher

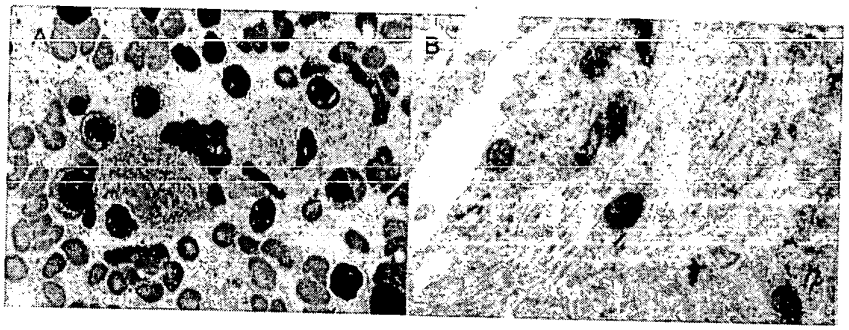

Figure 1. Light (*A*) and electron (*B*) micrographs (EMS) of Gaucher cells from bone marrow. Numerous cytoplasmic striations are present in (*A*), which under EM appear more tubular. These tubules are twisted bilayers of GC, phospholipid and small amounts of protein that are stacked. Most of these structures are unit membrane bound. A typical Gaucher cell is packed with these structures, whereas in macrophage/Gaucher cell precursors these are sparse.

cells (macrophage) may prove important to the pathogenesis of architectural bone disease in Gaucher patients.

Unlike visceral tissues, the CNS disease in the type 2 and 3 variants does not derive from accumulations of Gaucher cells nor large amounts of GC storage. Histologically, the major consistent finding has been the progressive loss of neuronal cells [27–31]. Gaucher-like glial derived cells are present within brain substance, but their numbers are not great and this finding is inconsistent. In comparison with GC, the accumulation of the GC-deacylated analogue, glucosylsphingosine, is greatly increased and has been proposed to be a toxic agent leading to neuronal toxicity [32–34]. These findings indicate that the fundamental pathophysiology in the CNS and visceral tissues differs significantly.

Cytokines

The accumulated GC and glucosylsphingosine have been suggested to activate macrophages and induce inflammatory responses by releasing cytokines. Immunohistochemical studies of bone marrow biopsies and spleens of patients with Gaucher disease and chronic myeloid leukemia showed similar origins of Gaucher cells and pseudo-Gaucher cells, respectively. In particular, human leukocyte antigen (HLA)-DR antigens are expressed at higher levels in Gaucher cells compared with pseudo-Gaucher cells [35]. This level of expression suggested that cytokines might be increased in the Gaucher cell or in surrounding cells that interact with these macrophages [36]. Indeed, interleukin (IL)-6 and IL-10 are elevated in sera from patients with Gaucher disease [37]. Increased expression of IL-1β messenger RNA (mRNA) and a trend toward elevated tumor necrosis factor α (TNF-α) mRNA also was found in Gaucher disease patients [38]. A variety of other cytokines were variably elevated in Gaucher patient sera, including IL-1β, IL-1Ra, IL-6, soluble IL-2 receptor (sIL-2R) and transforming growth factor β (TGF-β) [39].

In summary, elevated cytokines have been variably increased in Gaucher cells, and plasma/serum from Gaucher patients. Furthermore, there is a trend to increasing levels of these cytokines in serum associated with disease severity. How these cytokines are triggered to be produced, what regulates their production and how they impact the pathogenesis of Gaucher disease remain unclear. In addition, the local concentrations and effects of elevated cytokines may have greater impact on the disease manifestations than is reflected by serum levels.

Molecular genetics

Gaucher disease is transmitted as an autosomal recessive trait. The 7.5-kb (11 exons) gene is located on chromosome 1q21 (see fig. 2) and encodes acid β-glucosidase (GCase, glucocerebrosidase, *N*-acyl-sphingosyl-β-D-glucose: glucohydrolase, EC 3.21.45). Importantly, many mutations causal to Gaucher disease appear to arise from gene conversion events with the pseudogene: this remains to be formally proven [40]. The GCase pseudogene is highly homologous to normal gene (96% identity), ~5 kb in length, and is 16 kb downstream from the functional gene [41]. The GCase pseudogene is transcribed, but no functional protein can result because of numerous mutations that generate stop codons [40–42]. The major differences between the functional and pseudogenes are the presence of several small deletions in introns 2, 4, 6 and 7, numerous exonic missense mutations, and a 55-bp deletion in exon 9 in the pseudogene. Several other genes surround the GCase gene [43–48] (fig. 2). The genomic structure in this region is dense and contains pseudogenes, indicating an evolutionarily recent duplication (fig. 2). Any functional relationships between these genes and the pathophysiology of Gaucher disease are, at present, speculative.

Two major and two minor haplotypes have been delineated at the GCase locus (GBA) [49]. The N370S and c84

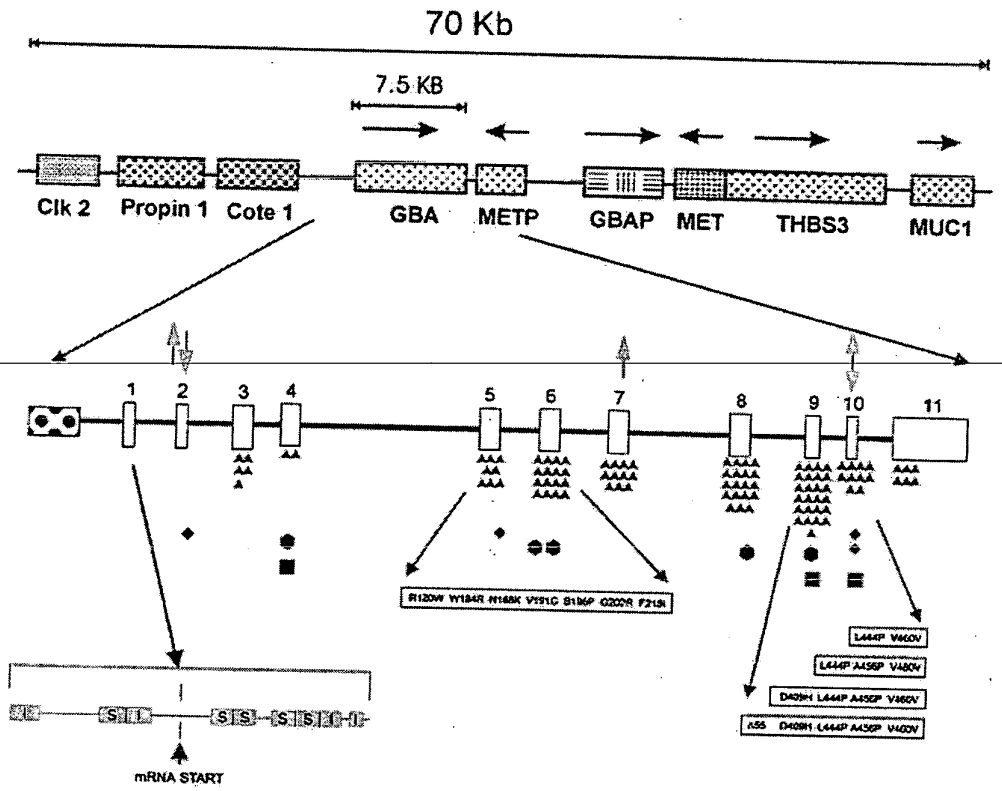

Figure 2. Schematic of the GBA locus and its surrounding genes on chromosome 1. Clk2, a gene resembling a serine/threonine kinase; propin 1, a gene of unknown function; cote 1, a gene of unknown function; METP, pseudogene of metaxin; MET, metaxin, a mitochondrial membrane protein; GBAP, pseudogene of GBA. THBS3, thrombospondin; MUCI, polymorphic epithelial mucin gene. The location of many GBA mutations is indicated throughout its 11 exons as follows: exonic missense mutations (▲), splice junction mutation (◆), large deletions (■), point deletions ( ), point insertions ( ), complex mutations (rectangles with text indicating multiple point mutations) and termination mutations (●). The rectangular block containing several mutations represents known 'complex alleles' that may have arisen from recombination with GBAP. Not shown is a complete gene deletion and a recombinant allele that includes pseudogene sequences spanning exons 2–11. In the lower left is an expansion of exon 1 showing putative stimulatory (S) and inhibitory (I) sequences for transcription of GCase.

ins G alleles are in linkage disequilibrium with the haplotypes. Using these data, the dates of separation and potential reoccurrence of the common Jewish mutation were assigned to 48 generations (N370S) and 55.5 generations (c84 ins G) [50]. Extragenic sequences of the glucocerebrosidase gene region also are polymorphic [51,52]. In addition, linkage disequilibrium of these haplotypes and an extragenic polymorphism was used to assess the dates of separation, and potential reoccurrence of the common Jewish mutations has assigned to be 40–1000 generations (1000–25,000 years) for N370S mutation and 50–4800 generations (1300–120,000 years) for the c84 ins G mutation [53]. This linkage indicates the recent origin of the N370S mutation. Their functional significance is unknown.

There are two upstream potential ATG start codons in the GCase full-length open (2.2–2.5-kb cDNA) reading frame. Both in-frame ATGs can function to produce active enzyme in cultured fibroblasts [54–56]. The preferential use of either ATG in various tissues [illegible], nor is the potential physiologic relevance of their use. Upstream TATA and CAAT boxes have been identified by S1 analysis, but their exact function has not been defined [41,57]. Also, positive and negative regulatory sequences were detected in the first exon and intron in tissue culture systems [58,59] (fig. 2). Their in vivo physiologic importance is not known. By in situ hybridization with antisense GCase mRNA, differential expression was shown particularly within the brain of mice [60]. During nearly all of embryonic and fetal development GCase mRNA is at a low level in most visceral tissues. In skin epidermis, the expression becomes high shortly before birth and is maintained throughout adult life. In comparison, a generalized low-level expression of GCase mRNA in the brain of early embryos transforms shortly before birth into specific high-level expression in neurons of the

cerebral cortex, hippocampus, basal ganglia, dentate nucleus and in the Purkinje cell layer of the cerebellar cortex. This pattern of expression is maintained throughout adult life. Studies are needed to confirm the relevance to humans, but these results indicate the need to examine the role of gene expression control in the expression of the phenotypes.

Mutations

Nearly 200 different mutations at the GBA locus have been identified in patients with Gaucher disease. These mutations include missense, termination, deletion and insertions [61–66]. Most of these are rare and/or private mutations but several have significant frequencies. The occurrence of pseudogene-like mutations in the functional gene from affected patients is important for diagnostic and potentially mechanistic studies. Several point mutations including L444P – the most common mutation worldwide – arise from apparent recurrent gene conversion or other rearrangement between the functional and pseudogenes. Similarly, Gaucher disease alleles containing 2, 3 or more point mutations and/or deletions and insertions, identical to those in the pseudogene, have been found around the world in apparently distantly related populations [65, 67, 68]. These observations provide credence to the 'gene conversion' origin of many of the common alleles. In comparison, the most frequent Gaucher disease allele in the Ashkenazi Jewish population, N370S, does not appear in the pseudogene, and is likely due to a founder effect [50]. The spontaneous mutational event that established this allele appears to have occurred or entered the European population nearly simultaneously with the migration of Middle Eastern Jews to that continent. The perpetuation of the N370S alleles in the Ashkenazi population likely resulted from sociopolitical forces, but heterozygote selective advantage cannot be excluded. Table 2 summarizes the frequencies of the more common alleles in Jewish and non-Jewish populations. The biochemical effects of [illegible] point mutations are discussed later.

Table 2. Allele distribution in Gaucher disease Type 1 Patients.

Allele	Jewish (%) (n = 1160)*	Non-Jewish (%) (n = 419)
N370S	71.82	43.6
c84 ins G	11.20	0.2
L444P	2.84	25.6
IVS 2^{+1} g→a	1.72	0.7
Rec*	1.42	3.5
Alleles detected	89	74
Other Alleles	11	26

* n = total alleles. Rec, recombinant alleles (RecNci and RecTL) that include the L444P missense mutation and at least one additional pseudogene mutation.

Genotype and phenotype correlations and threshold effects

Beyond the obvious clinical and personal impact, elucidation of genotype and phenotype relationships has significant biological implications. In particular, the delineation of specific organ involvement or variation in overall phenotype could reflect specific functions or levels of GCase needed for normal metabolism and/or the participation of other loci in the expressivity of the trait(s). To date, the genotype and phenotype relationships in Gaucher disease have been restricted by population numbers to the major mutation alleles, N370S, L444P and D409H. Review of all reported cases (~400) and the personal experience of one of us (G.A.G.) shows that the presence of the N370S allele, in the homoallelic state or as the heteroallele with another mutant GCase allele, absolutely correlates with type 1, nonneuronopathic Gaucher disease. The recent soft correlations with 'Parkinsonian-like' neurologic disease in such patients [69–71] requires careful attention since a subpopulation of nonneuronopathic Gaucher disease patients may be predisposed to such neuronopathic manifestations. By comparison, L444P/L444P and D409H/D409H are highly associated with development of neuronopathic disease at some time in life. In addition, a striking difference in tissue involvement is present in L444P/L444P vs. D409H/D409H patients [4, 72, 73]. The neuronopathic patients with L444P/L444P have varying degrees of visceral (hepatic, splenic, bony and lung) disease, but do not have the cardiac valvular calcifications, cataracts and hydrocephalus, and milder other visceral involvement characteristic of D409H/D409H. The clinical neuronopathic involvement in L444P/L444P and D409H/D409H patients is quite similar. Also, both mutations produce catalytically defective, unstable proteins with similar properties [74]. Thus, the bases for the discrepancies of visceral phenotypes of patients with these two genotypes are not explained by the known biochemical properties of the mutant enzymes and remain an intriguing area for investigation. Also, well characterized L444P/L444P patients are known to us, and reported by others who have apparent nonneuronopathic disease into the 3rd decade [18, 75]. Even if such patients do develop neuronopathic deterioration later in life, other major influences must impact the rate of progression.

The Swedish population of Gaucher disease patients is instructive in this regard. This variant of Gaucher disease derived from a single founder couple in the 17th century in the Norbottnian region of Northern Sweden [18, 75, 76]. A single GCase genotype, L444P/L444P, is present. However, the variation in phenotype ranges from severe infantile neuronopathic and visceral disease to milder variants with neuronopathic disease onset in the 4th to 6th decades. Since the environment is relatively similar, this major variation in phenotype must have a significant genetic basis, i.e. modifier genes.

The variation among Gaucher disease patients with N370S alleles also is great. Shown in figure 3 is a distribution of ages of onset and diagnosis of symptomatic Ashkenazi Jewish patients with N370S/N370S or N370S/'other mutant allele' genotypes [77]. Allowing for the vagaries of 'age at onset' and some genotyping errors, the results correspond closely to those from a larger, more diverse, population [78]. Importantly, these data represent patients from several European and American geographical regions. Similar relationships are present when hepatic or splenic volumes are used as parameters, with smaller organs being present in N370S/N370S patients [14, 77, 79]. Clearly, the N370S/N370S is associated with a less severe phenotype [3, 78]. Indeed, based on heterozygote frequencies, a paucity (~50%) of N370S/N370S genotypes are represented among the 'significantly' symptomatic patients with N370S alleles [3, 11]. The N370S/'other GCase mutant allele' phenotypes are more severe, and have ages of onset from 1 to 2 decades earlier than those with N370S/N370S [77, 78]. Since these data have been collected from widely different geographic and cultural milieus, the environmental impact on phenotype will be minimized, albeit not eliminated. Thus, the variation within each genotypic group probably represents genetic influences, i.e. modifier genes. However, the great variation between the N370S/N370S genotype and the other genotypes represents a major impact of the primary, GCase, genetic mutation.

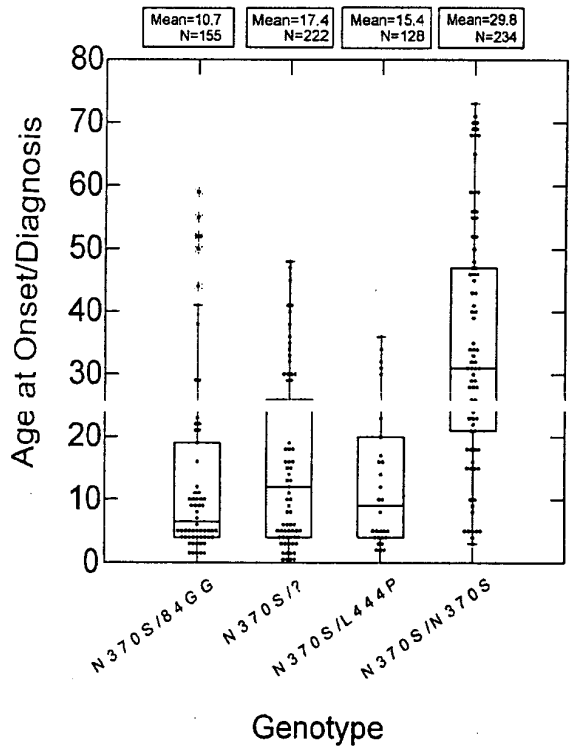

Figure 3. Age at onset and diagnosis for the patients with differing genotypes. The data were obtained from medical records and/or histories of symptomatic Ashkenazi Jewish patients. The boxes represent the median and first quartile boundaries. Each dot represents a single patient.

The N370S allele expresses a defective protein with substantial (~10–20% of normal) catalytic activity. In comparison, the c 84 ins G and IVS+2 alleles are null, and the L444P allele produces a highly defective protein with very little (but not zero) catalytic activity. Thus, most of the N370S/ 'other mutant alleles' or N370S/L444P patients have functionally half or less, respectively, the residual enzyme activity in vitro compared with N370S/N370S patients. Notably, the L444P/L444P patients have an average age at onset/diagnosis of 2.3 years [14].

The variable expressivity of the phenotypes would appear to be determined as follows: (i) For whole body phenotype, the residual activity of the mutant GCase sets the boundaries of potential variation in response to other influences. For example, an N370S/N370S patient could have a very wide range of potential response to other genetic polymorphisms or environmental stimuli and factors because of the amount of available residual enzyme. These stimuli and factors could influence substrate influx and production and/or mutant enzyme stability or activity. Thus, great variation in expression is expected. In comparison, the much lower residual activity in L444P/L444P patients would lead to much lower toleration of endogenous (genetic) or exogenous variations, with most being significantly deleterious. The least phenotypic variation, would be anticipated in phenotypes resulting from a null mutation that would allow for little, if any, response to varying input. (ii) For tissue-specific phenotypes, the effects of the mutant enzymes may vary with the tissue. For example, the compositions of glycosphingolipids present in cardiac valves, skin or brain differ and may interact with the mutant enzyme differently. GC in brain has shorter fatty acid acyl chains ($\sim C_{16-18}$) compared with that from visceral sources ($\sim C_{18-21}$) [80–82]. In comparison, skin has very long fatty acid acyl chains ($\sim C_{27-30}$) [83–87]. If in vitro differences in substrate specificity are reflected in vivo, some tissues could be 'protected' or not from disease manifestations even if the level of in vitro residual activities were identical with a particular substrate for the two mutant enzymes.

Recently, we have created mice with GCase point mutations, including N370S. Surprisingly, the N370S/N370S genotype in mice is lethal, with death due to disruption of the skin permeability barrier and the development of the severe ichthyosis [88]. This is identical to the GCase null mouse created nearly a decade ago [89]. In vitro initial characterization indicates that the mouse

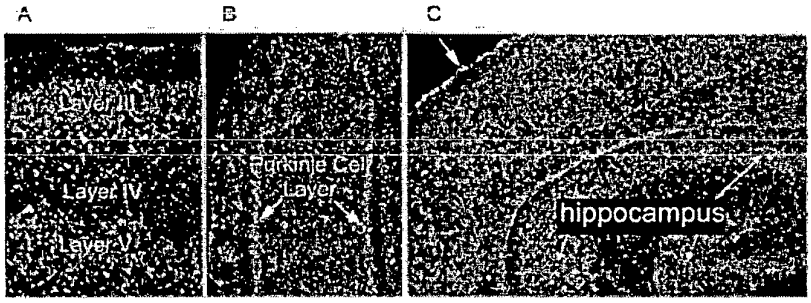

Figure 4. Expression of GCase mRNA in adult mouse CNS. Fluorescence in situ hybridization was performed with antisense GCase mouse RNA as probe. GCase mRNA signals are white to pink in the dark field. (*A*) Cerebral cortex neurons of layers III and V (arrowheads) have higher signal than those in layer IV (darker region in between III and V. (*B*) Cerebellar cortex with highly stained Purkinje cell layer. Both Purkinje (arrowheads) and granular cells have more intense signal than the surrounding tissues. (*C*) Intense staining of the hippocampal pyramidal cells (h). The meninges also have high signals (arrowheads).

N370S protein has very similar properties to the human N370S. However, more 'natural' assays with GCs containing very long chain fatty acid will need to be tested to evaluate the differences between the human and mouse wild-type and N370S enzymes to explain this phenotype.

Biochemistry and enzymology

Control of GCase expression

Although GCase is considered a housekeeping gene, its expression is controlled at the transcriptional, translational and posttranslational levels. The in vivo promoters that convey specificity to GCase expression have not been defined. However, developmental and tissue-specific GCase mRNA expression has been documented, particularly within regions of the brain [60]. In mice through about two-thirds of pregnancy, GCase mRNA expression is relatively low and ubiquitous [60]. Near term higher levels of expression are noted in neurons of the cerebrum, cerebellum, brain stem and spinal cord. This expression reaches a maximum following birth and into adulthood. Striking expression is observed in Purkinje cells and dentate nuclei of the cerebellum (fig. 4) and in the hippocampus. The differential signals result from increased concentrations of cells with relatively high expression surrounded by areas of much lower cell densities, rather than only high levels of mRNA per cell. General visceral expression is much lower and ubiquitous, except for higher levels in the epidermis of the skin. Thus, significant control of the timing of GCase mRNA expression is evident, and expression in neurons of the CNS and epidermis is higher than that in other cellular types. The temporal control promoters remain to be defined in vivo.

During overexpression studies of GCase mRNA in several mammalian cell types, a discrepancy (> 100-fold) was detected between mRNA expression and GCase protein production. This was due to the constitutive presence of a translational control protein (TCP), M_r ~80,000–90,000 that bound to double stranded RNAs [90–92]. The protein is identical to a variant of NF90, a protein originally implicated in the control of adenovirus gene expression following infection of cells. This protein binds specifically to a 180-nucleotide region of GCase mRNA and prevents its interaction with polysomes, and therefore translation cannot be initiated. In addition, TCP80/NF90 can be phosphorylated by the classical protein kinase C (PKC) pathway, and deficient phosphorylation of TCP80/NF90 facilitates GCase mRNA translation inhibition. The exact role of this protein in the control of GCase steady-state levels is unknown, but such inhibition of translation clearly impacts the overexpression of this protein for therapeutic purposes. Elimination of the translation inhibition by TCP80/NF90 may allow for substantial increases in protein expression and enhanced secretion.

Posttranslational modifications do not normally play a great role in the control of GCase expression and maintenance of steady-state levels. However, occupancy of the first glycosylation site is essential to GCase activity, but not proteolytic stability [93]. In comparison, occupancy of the other four glycosylation sites are not critical to activity [93]. This is likely due to the maintenance of hydrophilicity in this region, and vectorial cotranslational glycosylation may be important to disulfide bond formation between residues C4, C16, C18 and C23. Thus, mutations in this region may have global effects on protein folding if either glycosylation or disulfide structure is affected.

Biochemistry and cell biology

GCase is a membrane-associated lysosomal β-glucosidase composed of 497 or 496 amino acids in the human or mouse, respectively [56, 94, 95]. The enzyme is a

glycoprotein that contains four of five occupied N-glycosylation sequences [96]. The occupied sites are in the N-terminal 60% of the sequence, while the unoccupied site is in the COOH-terminal 10% of the protein. The seven cysteines are at residues C4, C16, C18, C23, C128, C248 and C342. The first four cysteines participate in disulfide formation, while that at residue C128 is free and those at C248 and C342 may be free, although this is not resolved [E. Ponce and G. A. Grabowski, unpublished observation]. Occupancy of the first N-glycosylation site at N19, A20, T21 is essential for the development of a catalytically active conformer, probably by directing disulfide formation. Mutagenesis studies showed that substitution of C4, C16, C18 or C23 with serine leads to catalytically inactive proteins as a result of disulfide disruption. Similarly mutated enzymes at residues C128 or C248 retain complete and partial activity, respectively. Substitution at C342 with glycine leads to a catalytically defective protein due to its proximity to the catalytic nucleophile at residue 340 [97].

GCase is synthesized, its leader peptide cleaved upon trans-ER (endoplasmic reticulum) membrane passage, and is cotranslationally glycosylated in the ER with subsequent sequential oligosaccharide modifications with movement through the cis, mid and trans Golgi [98–100]. The enzyme is not phosphorylated, nor does it contain mannose-6-phosphate residues for lysosomal sorting [101, 102]. Little GCase is normally secreted from cells, but significant amounts are secreted in overexpressed states [90, 103, 104]. This finding implies a saturable sorting system for delivery of GCase to the lysosomal compartments [101]. Normal human GCase does not contain any of the recognized COOH-terminal hydrophobic signals used for targeting of lysosomal membrane proteins [105]. However, newly synthesized GCase sorts to the lysosome in an unglycosylated state following treatment of cells with tunicamycin [101]. This treatment also does not appear to alter the degree of membrane binding of GCase as assessed by saposin activation. Membrane attachment is important for the survival of GCase in cells. In overexpressed systems, in which significant amounts of enzyme remain free in the lysosomal lumen, the half-life of the free enzyme (non-secreted) protein is much reduced compared with that of the membrane bound form [101]. Clearly, the association of the enzyme and membrane is essential for maintaining sufficient steady-state amounts of enzyme activity for normalization of GC flux through the lysosomal compartments. The implications of these observations for enzyme and gene therapies are discussed below.

Once delivered to the lysosome, GCase becomes bound to the inner membrane surface of this organelle. The enzyme is not an integral membrane protein with transmembrane domains, but is tightly membrane associated. No evidence is available to show that GCase will associate with the plasma membrane of cells, other than through specific oligosaccharide receptors, i.e. the mannose receptor used for enzyme therapy. Binding to the inner lysosomal membrane is thought to be mediated by resident phospholipids, although binding to a protein receptor has not been formally excluded. Using purified GCase, artificial liposomal membranes, pure or mixed phospholipids are essential activators of the enzyme's hydrolytic activity, i.e., in the absence of such lipids GCase is inactive. Negatively charged head groups on these lipids, e.g., phosphatidylserine, phosphatidic acid, phosphatidylinositols, lysobisphosphatidic acid [106, 107], are required for the activation effects. GCase also requires such negatively charged phospholipids (NCPs) to have an unsaturated fatty acid acyl chain covalently attached or in proximity, i.e. free oleic acid or present on another contiguous non-NCP phospholipid, for activation effects in liposomal systems [106, 107]. Phosphatidylcholine has no activation properties [108, 109]. Spectral studies using intrinsic GCase tryptophan fluorescence show a λ blue shift and an alteration in secondary structure of GCase by circular dichroism upon binding of the enzyme to such NCP membranes [107]. This binding is fast, submicrosecond time frame, and dissociation from the membrane cannot be detected within 12 h [X. Qi and G. A. Grabowski, unpublished observation]. Binding is tight, has specific structural requirements (albeit stereospecificity has not been shown) and is associated with reconformation of GCase into an active form. These and the above metabolic labeling analyses show that membrane attachment is key to enzyme stability (proteolytic) and full catalytic activity within cells. The actual NCP(s) that plays these roles in vivo is (are) unknown. Although a potent activator, phosphatidylserine is not present in significant amounts in the inner lysosomal membrane. Indeed, the asymmetric distribution of this lipid to the inner leaflet of the plasma membrane is maintained topologically through the endocytic pathway, and a 'flip' would be required for phosphatidylserine to be a major physiologic activator. Lysobisphosphatidic acid and phosphatidylinositol phosphates are prime candidates since they are resident in the interior of lysosomal compartments [110, 111]. Lysobisphosphatidic acid is resistant to phospholipases [110, 112] and could provide a relatively 'stable' group of binding sites for GCase.

Additionally, GCase membrane attachment in the lysosome is important in understanding how the enzyme gains access to insoluble lipid substrates (GC) as they are presented to the late endosomal/lysosomal compartments. Such understanding might provide insight into the aberrant hydrolysis of GC and other substrates by mutant enzymes in Gaucher disease and the development of newer therapeutic approaches. In the phagocytic pathway, glycosphingolipid substrates cross the plasma membrane,

enter the endosomal system, pass through the multivesicular body (MVB) and enter the lysosomal compartment. Topologically, access of GCase to GC would require lysosomal luminal orientation of GC, potentially by first being oriented on the outer surface of the small vesicles of the MVB. Alternatively, GC could be incorporated into the lysosomal membrane through a fusion/hybrid compartment of late endosomes and lysosomes. This possibility seems less likely since it requires preferential or exclusive sorting of GC to the lysosomal membrane and away from the endosomes. Such a degree of specificity might occur through lipid rafts [113–116], but the mechanism is obscure. The delivery of GC-containing MVB vesicles to the lysosomes as a surface for GCase action does not require such a high degree of specific sorting, since only the formation of a hybrid compartment is needed. The MVB small vesicles, containing many lipids, could be incorporated into the lysosomal compartment. Since GC is not present in aqueous solution and must be presented in a membrane form, either of these mechanisms requires that membrane bound GCase 'confront' GC in a membrane. Available data indicate that GCase requires membrane attachment, interaction with NCP and an activator protein (saposin C) for hydrolytic activity [106,107,117]. GCase membrane attachment appears tight and a 'scooting mode' of hydrolysis is likely, i.e. GCase functions only by hydrolysis of GC in the interfacial mode with both enzyme and substrate on the membrane surface at least in vitro. This implies that GCase and GC move to confront each other in the lysosomal membrane or that GCase or GC must move from the MVB small vesicles to another membrane to confront each other. Based on current knowledge of GCase, this enzyme, once bound, does not dissociate from the membrane. Consequently, only newly synthesized GCase that might be freed from the lysosomal membrane, albeit this is not known, could interact with MVB, or collision and/or restructuring of the small vesicles would be needed for GCase to access the substrate. Removal of the substrate from the membrane for presentation to GCase is unlikely, since, contrary to saposin B's mechanism with more water-soluble glycosphingolipids, saposin C requires GCase at the NCP interface for activation effects [118]. This interaction is independent of the presence of GC or other substrates [106]. This is summarized in figure 5.

Such mechanistic insights are needed to understand the potential for mutant enzyme interactions and potential in situ modifications that could be therapeutic. For example, the N370S enzyme in vitro has greater activation effect by NCPs than the wild-type counterpart [108,119,120]. Since this mutant enzyme protein is present in normal

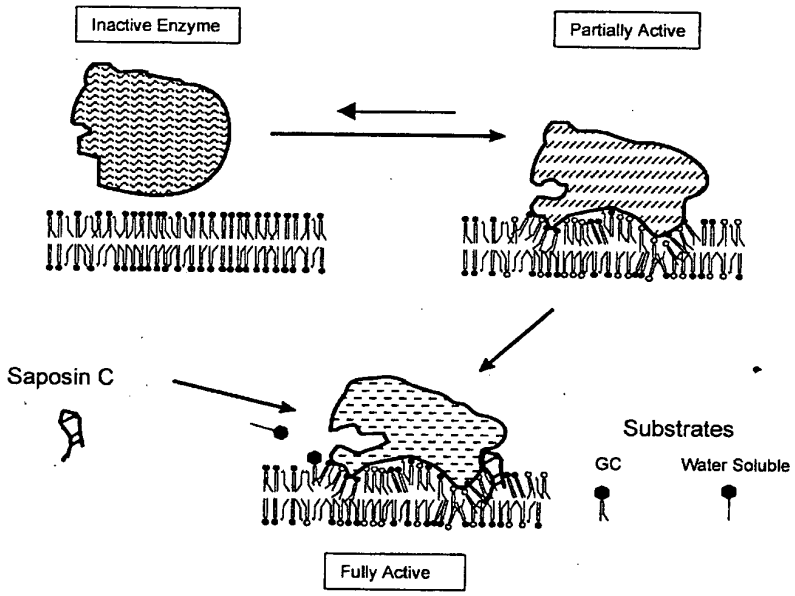

Figure 5. Schematic of GCase and its activation upon association with lysosomal membrane. In the soluble form, GCase is inactive. Upon attachment to negatively charged phospholipids (NCP, filled head groups), GCase undergoes a conformational change with realignment of residues in the active site. The enzyme then has catalytic activity. This attachment step is slowly reversible as indicated by the arrows. Saposin C is soluble and attaches to the GCase/NCP complex and induces a further conformational change. This leads to a fully active GCase that is capable of cleaving membrane-bound and water-soluble substrates. In the absence of saposin C, in vivo, GCase has low-level activity. In vitro and, potentially, in vivo, interaction of saposin C may require GCase bound to NCP: i.e. NCP, may make GCase conformation acceptable to saposin C.

amounts in cells from humans with Gaucher disease, the potential exists to enhance the activity above a threshold for corrections of GC flux through cells. In addition, this enzyme attaches to NCP membranes and occupies binding sites in NCP liposomes. This suggests a potential for competition between different enzyme forms for binding sites within the lysosome and, depending upon the mechanism of enzyme interaction with lysosomal substrates, could interfere with therapeutic supplementation by enzyme.

Pathogenesis

Two competing hypotheses for the pathogenesis of Gaucher disease include (i) Gaucher cells as relatively inert long-lived space-occupying lesions or the constipated lysosome hypothesis, and (ii) the transduction box hypothesis in which there is a deficiency of an essential signal that normally would leave the lysosome following cleavage of GC and have subsequent effects on cellular functions. These two hypotheses are not mutually exclusive, and the components may exist for both. The first has been assumed as a principle for more than 2 decades and envisions a passive role for the lysosomal storage material. Except for the lysosphingolipid hypothesis of Hannun and Bell [121], the second hypothesis has received little attention. This second hypothesis is different than the lysosphingolipid hypothesis since it assumes an active normal role for sphingolipid products that leave the lysosome, rather than a toxic role for deacylated analogues of glycosphingolipids. Lacking an animal model, for direct studies, pathogenic analyses of Gaucher disease have focused primarily on static pathologic samples that may reflect end-stage organ or cellular disease, rather than an early pathophysiologic processes. The constipated lysosome hypothesis is based primarily on the apparent accumulation of engorged macrophages in various tissues that could result from either filling up of the macrophages with GC, or accumulation of GC with resultant proliferation of macrophages. This is a relatively late event in tissues. Evidence for either is lacking, and mitotic figures of macrophage precursors as they enter various organs have not been obvious. Thus, lacking, direct analyses by markers of cellular proliferation, the space-occupying lesion hypothesis cannot be formally excluded. Similarly, the active hypothesis requires additional in vivo data for support. However, for these authors, this is a more attractive working hypothesis. Developing evidence supports elaboration of substances by activated macrophages due to GCase defects including cytokines, proteases and other markers such as angiotensin-converting enzyme and chitotriosidase. Several of these are secreted or lost from macrophages when activated by exogenous agents during immunologic responses. Analysis of bony lesions in Gaucher disease with particular attention to the cortical bone indicates an active metabolic process whereby bone structure is lost [122]. Thus, a reasonable hypothesis would include activation of the macrophage system by some lipid component that would normally move from the lysosome to the cytoplasm with subsequent enhancement of a cascade of transcriptome and proteome effects. A volumetric effect of the engorged lysosomes might also lead to cellular activation, but there is no evidence for this. It should be noted that the lysosphingolipid hypothesis and/or the activation hypothesis might be relevant to the CNS disease in which neuronal cell death is the major pathologic consequence, and not macrophage proliferation and cellular accumulation.

The overall hypothesis includes the following: a tissue macrophage becomes bound, and GC presented to the lysosome through phagocytosis cannot be broken down and accumulates, but the major event is the lack of effector egress from the lysosome. This effector is unknown. However, ceramide seems an attractive candidate since it has been shown, at least when generated from sphingomyelin in the plasma membrane, to become phosphorylated and lead to a series of events including apoptosis. It could be envisioned that the loss of this effector would lead to increased production of cytokines by the loss of feedback signals to the nucleus of active agents, such as cytokines or other markers of macrophage activation, and local hyperplastic or hyperfunction effects on macrophage-function. This has been demonstrated in the lysosomal acid lipase deficient mouse in which macrophage-colony stimulating factor (mCSF) is upregulated due to a deficiency of a fatty acid being blocked from egress from the lysosome [123]. Thus, lipids could have repressor effects either directly as signals or mediated through other events, such as the plasma membrane composition, producing activation of the macrophage and proliferation of these cells. The continual presentation of GC to the macrophages and the inability to digest this lipid to its components can be viewed as a secondary event. The primary event would be the lack of effector egress and expansion of the macrophage space into which GC could accumulate. This dysregulation of such activators would lead to more generalized macrophage production and increased synthesis of macrophage activation markers as described above. Furthermore, the elaboration of such compounds would produce hyperplasia of the macrophage compartment, and a self-perpetuating vicious cycle would result due to the lack of a signal to turn off the continuous production of macrophages. Whether the presence of additional macrophages alone would have a continuing detrimental effect or whether these secondary cytokines or other pathologic agents would have an effect requires direct experimental support. The availability of viable mouse models with Gaucher disease provides the opportunity to address such hypotheses to then close the

loop between the molecular biology and the cell biology and pathogenesis of Gaucher disease [88]. Through such studies of transcriptome and proteome analysis, additional targets for improved or adjuvant therapies would become available potentially to improve current responses to therapy.

Enzyme and gene therapy

The outstanding accomplishment by Dr Roscoe Brady and co-workers [124–126] of effective and safe enzyme therapy for Gaucher disease has been reviewed extensively [12]. The readers are referred to these references for details. Succinctly put, after a decade of enzyme therapy, the population of Gaucher patients have had major improvements in health and reversal of many aspects of their pathology. The treatment of CNS and established bone disease remain major challenges for clinical researchers. The improvement of therapeutic efficiency and reduction of cost are major challenges for the next decade. Gaucher disease type 1 is also a prime candidate for hematopoietic stem cell gene therapy since the major primary pathology derives from the bone-marrow-originating monocyte and macrophage cells. Recent successes in gene therapy for immunodeficiency diseases [127] indicate that progress is being made in this therapeutic strategy.

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Exhibit C

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Supporting Online Material

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Materials and Methods
Fig. S1
Table S1

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SCNM1, a Putative RNA Splicing Factor That Modifies Disease Severity in Mice

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The severity of many inherited disorders is influenced by genetic background. We describe a modifier interaction in C57BL/6J mice that converts a chronic movement disorder into a lethal neurological disease. The primary mutation (med^J) changes a splice donor site of the sodium channel gene *Scn8a* ($Na_v1.6$). The modifier mutation is characteristic of strain C57BL/6J and introduces a nonsense codon into sodium channel modifier 1 (SCNM1), a zinc finger protein and a putative splice factor. An internally deleted SCNM1 protein is also predicted as a result of exon skipping associated with disruption of a consensus exonic splicing enhancer. The effect of the modifier mutation is to reduce the abundance of correctly spliced sodium channel transcripts below the threshold for survival. Our finding that genetic variation in a putative RNA splicing factor influences disease susceptibility in mice raises the possibility that a similar mechanism modifies the severity of human inherited disorders.

About 10% of human disease mutations alter pre-mRNA splice sites. Genetic variation in proteins that regulate splicing has been predicted to result in trans-acting modification of disease severity for splice-site mutations (*1*, *2*). With the use of the mouse as a model, we describe here an example of genetic interaction between a putative splicing factor and a splice-site mutation in a neuronal sodium channel gene. *Scn8a* encodes the sodium channel $Na_v1.6$, which is localized on dendrites and axons throughout the nervous system and concentrated at nodes of Ranvier in myelinated axons (*3*). Mutations in mouse *Scn8a* cause inherited movement disorders that range in severity from tremor to ataxia, dystonia, and juvenile lethality (*4*–*6*). The severity of the hypomorphic allele $Scn8a^{med^J}$ is determined by the unlinked modifier gene *Scnm1* (*7*).

$Scn8a^{med^J}$ (med^J) contains a mutation in the splice donor site of intron 3 (Fig. 1A). The abundance of *Scn8a* mRNA is normal in med^J homozygotes, but there is a mixture of correctly and incorrectly spliced transcripts, with predominance of the incorrect transcript (*6*–*8*). This transcript skips exon 2 and exon 3 and encodes a truncated, nonfunctional protein (Fig. 1A). The severity of the med^J movement disorder is dramatically affected by strain background. This effect was mapped to a single Mendelian locus, sodium channel modifier 1 (*Scnm1*), which determines the proportion of correctly spliced transcripts and hence disease severity (*7*). C3H and other common inbred strains carry the resistance allele of *Scnm1*. Resistant med^J/med^J mice produce 10% correctly spliced transcripts, exhibit a progressive disorder with dystonia and ataxia, and live for >1.5 years (*8*). C57BL/6J mice carry the recessive susceptibility allele of the modifier. Susceptible med^J/med^J mice produce only 5% of correctly spliced transcripts, become paralyzed, and do not survive beyond 1 month (*7*, *8*). Reduction of correctly spliced transcripts to 5% in $med^J/-$ compound heterozygotes is also lethal (*8*).

We previously mapped the *Scnm1* locus to a 950-kb region on mouse chromosome 3 containing about 34 candidate genes and a recombination hot spot (*9*). To identify *Scnm1*, we compared the finished genomic sequence of the nonrecombinant region from strain C57BL/6J (*9*) with the human orthologous sequence on chromosome 1q21 (*10*). This sequence comparison identified a premature stop codon, R187X, in the C57BL/6J allele of the predicted gene MGC3180 (*11*). The wild-type MGC3180 gene in strain C3H contains seven exons and encodes a protein of 229 amino acids (Fig. 1B). The stop codon in C57BL/6J removes the C-terminal 43 residues (Fig. 1B).

Reverse transcription polymerase chain reaction (RT-PCR) of RNA from C57BL/6J mice with primers in exon 5 and exon 7 resulted in amplification of the normal 330-base-pair (bp) product and a unique 135-bp product that lacks exon 6 (Fig. 2A). The transcript lacking exon 6 retains an open reading frame and encodes a 164-residue protein, SCNM1Δ133-196. Analysis of the exon 6 sequence with software that detects exon splice enhancer sites (*12*) indicated that the C-to-T substitution in codon 187 destroys a predicted exon splice enhancer recognized by the arginine- and serine-rich protein ASF/SF2 (Fig. 2B). C57BL/6J mice are predicted to produce two abnormal proteins, one that is prematurely truncated at residue 186 and a smaller 164-residue protein, SCNM1Δ132-196, that lacks the residues encoded by exon 6 (Fig. 1B).

We determined the strain distribution of the wild-type and R187X alleles by amplifying exon 6 from genomic DNA of 36 inbred strains and digesting the product with the diagnostic restriction enzyme Hpy99I. The wild-type allele was present in 31 strains (fig. S1), including all five strains known to carry the resistance allele of *Scnm1* (*9*). The R187X allele was found only in the closely related C57 and C58 strains, suggesting that the mu-

Table 1. Rescue of C57BL/6J-med^J/med^J mice by transgenic expression of wild-type SCNM1. Rescue is indicated by the survival of transgenic mice beyond 40 days of age and by their typical dystonic phenotype. B indicates C57BL/6J; H, C3H.

Scn8a genotype	*Scnm1* genotype	Line	No. surviving >40 days	Lifespan (to present)	Full-length *Scn8a* transcript relative level
med^J/med^J	BB	C57BL/6J-med^J	0/11	30 ± 4 days	1.0 ± 0.2 (n = 3)
med^J/med^J	BB	Tg405 (BAC)	6/6	>8 months	1.9 ± 0.1 (n = 3)
med^J/med^J	BB	Tg580 (cDNA)	6/7	>2 months*	2.1 ± 0.3 (n = 4)
med^J/med^J	BH	see (*8*)	see (*8*)	>8 months	1.9 ± 0.3 (n = 3)
med^J/med^J	HH	see (*8*)	see (*8*)	>8 months	1.9 ± 0.2 (n = 3)

*Killed at 2 months.

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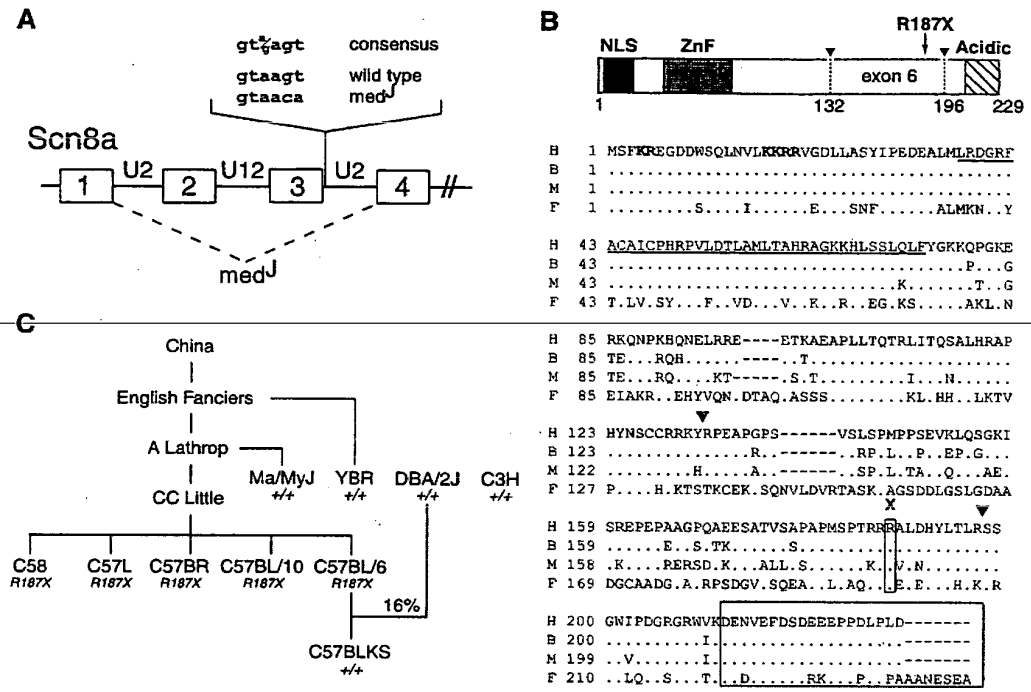

Fig. 1. Molecular variants of *Scn8a* and *Scnm1* interact to influence the severity of neurological disease. (A) A 4-bp deletion in the donor splice site of exon 3 in the *Scn8a*^{med} allele results in exon skipping (+5 to +8). The major processed transcript of the *Scn8a*^{med} allele skips both exon 2 and exon 3 because of the arrangement of U2 and U12 introns (*6*). (B) Protein domains and amino acid sequence of SCNM1 from mammals and fish. The mouse R187X mutation and the acidic domain are boxed, the nuclear localization signal (NLS) is in bold, and the zinc finger (ZnF) is underlined. H, *Homo sapiens*; B, *Bos taurus*; M, *Mus musculus*; F, *Fugu rubripes*. (C) The R187X mutation is shared by the closely related C57 and C58 strains. Genealogy suggests that the R187X mutation arose in the common ancestor to these strains about 80 years ago (*29*). The wild-type allele (+) was detected in 31 other inbred strains (fig. S1).

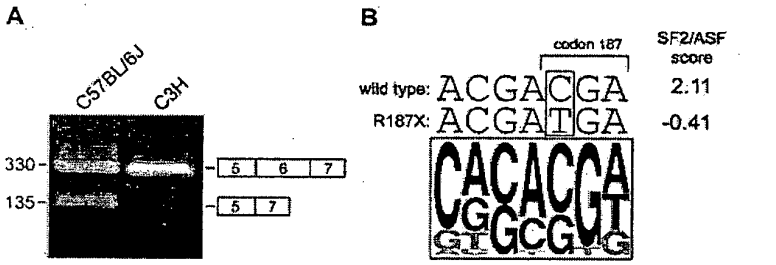

Fig. 2. Disruption of a consensus exonic splice enhancer in exon 6 of Scnm1 by the R187X [illegible] in strain C57BL/6J. (A) Aberrant splicing of *Scnm1* in C57BL/6J. The transcript that skips exon 6 is detectable by RT-PCR of adult brain RNA from the susceptible strain, C57BL/6J, but not from the wild-type (resistant) strain, C3H. (B) The SF2/ASF consensus exonic splice enhancer sequence is reprinted from (*30*) with permission. The nucleotide substitution in the mutant allele reduces the match to the consensus from a significant value of 2.1 to an insignificant value of −0.4, consistent with loss of function (*12*).

tation arose in a common ancestor of these strains about 80 years ago (Fig. 1C). Strain C57BLKS/J carries the DBA/2J haplotype in this chromosome region (fig. S1C).

To investigate whether the mutant allele of MGC3180 accounts for the disease susceptibility of strain C57BL/6J, we tested the ability of the wild-type allele to rescue the lethal phenotype. BAC (bacterial artificial chromosome) clone 26A24, isolated from the resistant strain 129S6, contains the wild-type MGC3180 gene as well as 10 flanking genes (fig. S2). Purified BAC DNA was microinjected into fertilized eggs from strain C57BL/6J. Transgenic founder #405 was crossed to the congenic line C57BL/6J-*med*^{J}/+, and the resulting transgenic, med^{J}/+ offspring were backcrossed to the congenic line. The *med*^{J}/*med*^{J}, Tg405–positive offspring had a typical resistant phenotype, including dystonic postures, demonstrating rescue by the BAC transgene (Table 1). The abundance of the wild-type MGC3180 transcript in brain RNA from line Tg405 was 50% that of the endogenous R187X transcript (fig. S3).

To confirm that MGC3180 is responsible for BAC rescue of the susceptible phenotype, we carried out a second rescue experiment with the use of a wild-type cDNA from strain C3H under the control of a ubiquitously expressed chicken β-actin promoter (*13*). The abundance of the wild-type MGC3180 transcript in brain RNA from transgenic line C57BL/6J-Tg580 expressing the cDNA construct was twice that of the endogenous R187X transcript, demonstrating that excess transcripts are tolerated (fig. S3). Transgenic mice from this line were backcrossed to the C57BL/6J-*med*^{J}/+ line as described above. C57BL/6J-*med*^{J}/*med*^{J}, Tg580 mice exhibited the dystonic phenotype and were rescued from juvenile lethality (Table 1). Thus, the predicted gene MGC3180 is the sodium channel modifier gene *Scnm1*.

To examine the splicing of the *Scn8a*^{med} pre-mRNA in the rescued transgenic mice, we determined the relative amounts of full-length and mutant transcripts with the use of a primer extension–chain termination assay (*8*, *14*). The proportion of correctly spliced *Scn8a* transcripts in both lines of transgenic mice was twofold higher than in nontransgenic *med*^{J}/*med*^{J} littermates (Table 1). The level of correctly spliced transcript in rescued transgenic mice was comparable to the level previously measured in mice with the wild-type (resistance) allele of *Scnm1* (*8*), demonstrating transgenic rescue of the C57BL/6J splicing defect. The fivefold difference in wild-type SCNM1 expression levels between Tg405 and Tg580 did not change the proportion of correctly

spliced *Scn8a*$^{med-J}$ pre-mRNA, indicating that SCNM1 is not rate-limiting for splicing in this concentration range.

Orthologs of *Scnm1* were identified in mammals, chicken, fish, and the urochordate *Ciona* with the use of public sequence databases, but no orthologs were detected in flies, worms, or yeast. Analysis of protein domains in the coding sequence revealed one C2H2 zinc finger, a basic nuclear localization signal, and a C-terminal acidic domain (Fig. 1B). The C2H2 zinc finger domain is 100% conserved in the mammalian orthologs of SCNM1 (Fig. 1B, underlined). Phylogenetic comparison of zinc finger sequences places SCNM1 within the U1C subfamily of RNA binding proteins that is commonly found in RNA-processing proteins (fig. S4). The U1C splice factor functions in recognition of splice donor sites (*15*, *16*). Two other subfamily members, SF3A2 and WBP4, are also involved in splicing (*17*, *18*). The homology between the zinc fingers in SCNM1 and established splice factors suggests that SCNM1 may also function in splicing.

The basic nuclear localization signal of SCNM1 matches the bipartite consensus $(K/R)_2 X_{10-12} (K/R)_3$ (*19*) (Fig. 1B). To examine subcellular localization, we fused the coding sequences of wild-type SCNM1^{229} and SCNM1^{186} downstream of the green fluorescent protein. Both fusion proteins were localized exclusively to the nucleus in transfected cells (fig. S5).

The R187X mutation in *Scnm1* impairs the in vivo splicing of *Scn8a*$^{med-J}$ in C57BL/6J mice. Because *Scnm1* is widely expressed in mouse embryonic and adult tissues (*20*), the mutation could also result in genome-wide changes in pre-mRNA processing, as observed for deletion of individual splicing factors in yeast (*21*). Impaired splicing of unlinked genes could be responsible for quantitative trait loci alleles and modifiers in strain C57BL/6J that map to this chromosome region, including vestibular dysfunction and alcohol preference (*22*–*24*). R187X could also confer general susceptibility to de novo splice-site mutations. Coisogenic C57BL/6J-Tg580 mice expressing wild-type SCNM1 will be useful for evaluating these predicted effects of *Scnm1*.

The effect of genetic background on the severity of inherited disorders has long been recognized (*25*), but only a few modifiers have been molecularly identified to date (*26*, *27*). Trans-acting splice factors have been suggested as one class of modifiers of the severity of human inherited disorders (*28*). *Scnm1* provides an example of this type of disease susceptibility through its trans-effect on the *Scn8a*$^{med-J}$ transcript. The role of human *SCNM1* in modulating splicing defects can now be tested with the use of linkage markers on human chromosome 1q21.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/301/5635/967/DC1
Materials and Methods
Figs. S1 to S6
References and Notes

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Loss of a Callose Synthase Results in Salicylic Acid–Dependent Disease Resistance

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Plants attacked by pathogens rapidly deposit callose, a β-1,3-glucan, at wound sites. Traditionally, this deposition is thought to reinforce the cell wall and is regarded as a defense response. Surprisingly, here we found that *powdery mildew resistant 4* (*pmr4*), a mutant lacking pathogen-induced callose, became resistant to pathogens, rather than more susceptible. This resistance was due to mutation of a callose synthase, resulting in a loss of the induced callose response. Double-mutant analysis indicated that blocking the salicylic acid (SA) defense signaling pathway was sufficient to restore susceptibility to *pmr4* mutants. Thus, callose or callose synthase negatively regulates the SA pathway.

Plants defend themselves from pathogens with a variety of chemical and physical defenses (*1*). As first reported in 1863 by de Bary, the most prominent physical defense is the rapid synthesis of callose, an amorphous, high-molecular-weight β-1,3-glucan (*2*). During fungal infections, callose is deposited in cell wall appositions (papillae) that form beneath infection sites and are thought to provide a physical barrier to penetration (*3*). One of the collection of *Arabidopsis* mutants resistant to the powdery mildew *Erysiphe cichoracearum*, *pmr4*, is resistant to other biotrophic pathogens (*E. orontii* and *Peronospora parasitica*), and resistance appears to act after the pathogen has penetrated the plant cell wall (*4*). Also, *pmr4* produces dramatically less callose in response to powdery mildew infection or wounding.

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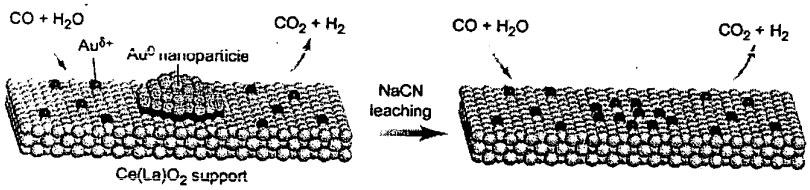

Actors and spectators. The rate of the water-gas shift reaction is not affected by leaching of metallic gold nanoparticles by a NaCN solution, indicating that the particles are spectators in this reaction. The active site involves supported cationic gold ($Au^{\delta+}$, red spheres; the exact charge is not known).

diameter, Valden *et al.* speculate that the unusual reactivity of supported gold results from a quantum size effect with respect to the thickness of the gold. The population of particles that are two atoms thick coincides with a maximum in catalytic activity.

However, the electronic properties of gold particles need not be solely related to quantum size effects. For example, Boyen *et al.* recently prepared silicon-supported gold particles by an inverse micelle synthetic strategy and chemical stabilization (*7*). The matrix surrounding the supported particles was removed through exposure to an oxygen plasma. Particles containing 55 atoms of gold, which are 1.4 nm in diameter, were completely resistant to oxidation by the plasma treatment. This extraordinary stability was attributed to the closing of the second atomic shell. Boyen *et al.* suggest that the resistance to oxidation of Au_{55} might contribute to the high catalytic activity of supported Au for CO oxidation. A related study also predicts an unusually high chemical stability of Au_{20} particles (*8*). However, it is not clear how the dissociation of oxygen molecules, a necessary step in the oxidation of CO, would occur on such inert particles.

Explanations of the high catalytic activity of gold particles on the basis of size alone neglect the importance of the underlying support. A growing body of research suggests a role of the metal-support interface. For example, in CO oxidation, oxygen might adsorb on the support whereas carbon monoxide might adsorb on the gold. Thus, the periphery of the gold particles could be the site for the oxidation reaction (*9*). Furthermore, because metal particles often have positively charged atoms at the metal-support interface, a unique role of cationic interfacial gold species has been proposed (*5*, *10*, *11*).

The elegant experiments of Fu *et al.* now reveal the critical role of cationic gold in the water-gas shift reaction (*1*). The authors deposited gold onto a La-doped cerium oxide support by either deposition-precipitation or coprecipitation and then calcined the material in air at 400°C. Most of the gold was reduced to metallic particles by the thermal treatment. A basic sodium cyanide solution was then used to leach the metallic gold from the catalyst surface (the same process is used to extract gold during mining operations). X-ray photoelectron spectroscopy showed that in a coprecipitated sample, the gold remaining on the support after leaching was exclusively ionic (see the figure).

Although the leaching process removed 85% of the gold, the catalytic activity per surface area was unaffected by the loss of metallic particles. These results suggest that ionic gold strongly interacts with the ceria support and is the active site for the reaction. Neutral gold (Au^0) metal particles are simply inert by-products of the catalyst preparation method. Results from similar experiments with Pt-loaded ceria also indicate that metallic Pt is a spectator and ionic Pt is the catalytic site.

Earlier this year, Guzman and Gates used Au^{3+} organometallic complexes to prepare MgO-supported gold particles of various sizes and tested the materials in the catalytic hydrogenation of ethene (*12*). After monitoring the chemical state of the supported gold with in situ x-ray absorption spectroscopy, they concluded that mononuclear Au^{3+} complexes were the active species and that Au^0 was inactive. These results extend the role of cationic gold to another support and reaction, but the general relevance of Au cations in catalysis remains to be discovered.

Cationic gold may be the key to using this noble metal in areas usually reserved for transition metals such as Pt and Pd. Because each cationic species is potentially an active site, the activity per gold atom may be very high compared with that of a supported metal particle, which exposes only a fraction of its atoms at the surface. Hopefully researchers will continue to explore the potential of other cationic, single-site systems.

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GENETICS

Modifying the Message

Joseph H. Nadeau

One of the pioneering discoveries in genetic research was that variation at one gene could modify the phenotypic expression of variants at another gene (*1*–*3*). We know now that phenotype modification is the rule rather than the exception and that genetic background strongly influences the expression of most genetic variants (*4*–*6*). The classical studies on phenotypic variation revealed that complex webs of interactions allow organisms to survive in the face of genetic and environmental stresses. The challenge now is to understand these networks in molecular detail. Key to this is identifying modifier genes and determining how they act on their targets. On page 967 of this issue, Buchner *et al.* (*7*) identify and characterize a naturally occurring genetic modifier of the mutation that causes neuronal defects in *med*^{J} mice. The modifier variant affects the severity of the phenotype by controlling splicing of the *med*^{J} pre-RNA transcript.

Mice that are homozygous for mutant alleles of *med*^{J} show symptoms ranging in severity from tremor to ataxia, dystonia, muscle atrophy, paralysis, and lethality (*8*). Mice with a severe phenotype have a fatal paralytic disease. They progressively lose function in their hindlimbs and usually die within several weeks. In contrast, mice with a mild phenotype can live normal lifespans with chronic movement disorders. Neuronal defects include deficient signal

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transduction at neuromuscular junctions, degeneration of Purkinje cells, and excess preterminal arborization.

The *med*^{J} mutation results from a short deletion (4 base pairs) that includes the 5′ donor splice site of exon 3 in the *Scn8a* sodium channel gene. The mutant transcript skips exons 2 and 3 and codes for a truncated protein. Both normally and abnormally spliced transcripts are produced, so that the overall abundance of the *Scn8a* transcript is normal. However, the normal transcript is found in much smaller amounts and accounts for at most 10% of the total *Scn8a* transcript (see the figure).

Ion transport is critical to many cellular functions, and genetic mutations that disrupt ion transport cause serious human diseases, such as cystic fibrosis, epilepsies, ataxia, and paralysis. The SCN family of proteins modulates transport of sodium into a wide variety of cells, including those in the kidneys, lung, muscles, and nerves, throughout development. SCN8A proteins are particularly active in muscle and neuronal cells, where they control the propagation of action potentials. The phenotype of *med*^{J} mutant mice is consistent with defects in sodium transport.

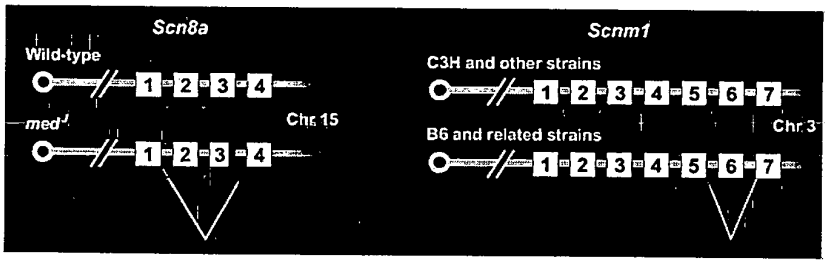
The severity of the *med*^{J} phenotype is dependent on genetic background (*9*). The milder phenotype is found in the C3H inbred strain and most other inbred strains, whereas the severe phenotype is found in C57BL/6J and closely related strains. The differences in phenotype correlate with differences at the molecular level; 10% of transcripts are normally spliced on the C3H background, whereas 5% of transcripts are correctly spliced on the B6 background (see the figure). Thus, rather subtle differences in transcript levels cause striking differences in phenotype. This suggests that viability requires a threshold level of normal transcript and that dropping below this threshold has profound consequences.

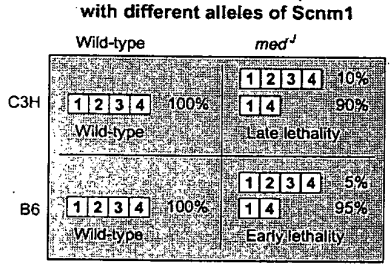
Using classic positional cloning methods, Buchner *et al.* (*7*) showed that the modifier that exacerbates the *med*^{J} phenotype in C57BL/6J strains encodes a zinc finger protein (SCNM1) that appears to act as a splice factor. The evidence is based on a combination of gene mapping, haplotype analysis, transcript characterization, and phenotype complementation in bacterial artificial chromosomes and complementary DNA transgenic mice. The variant allele of *Scnm1* involves a single nucleotide substitution that simultaneously destroys a splice enhancer site and creates a premature stop codon. Different truncated messenger RNAs (mRNAs) are produced, depending on which exons are included in the transcript and which are skipped. The *Scnm1* gene, which is highly conserved in chordates, belongs to the U1C subfamily of RNA binding proteins that play a role in recognition of splice donor sites. This provides compelling evidence that the modifier gene has been correctly identified.

Many disease mutations in humans and other species disrupt pre-mRNA splicing (*10*, *11*), and a rapidly growing body of evidence shows that various aspects of RNA biology, including splicing, are common targets of phenotypic modifiers (*11*). Mutations that affect the invariant splice-site sequences give no normal transcripts and thus often have severe phenotypic effects. By contrast, mutations in variant motifs that include splice enhancers and splice silencers produce a mixture of normal and truncated transcripts and thus tend to have more modest and more variable effects. Modifier genes can act on the latter group by modulating the ratio of normal to abnormal transcripts. There is potential to design drugs that target modifier proteins and thus modulate the level of normally spliced transcripts (*11*).

The Buchner study is perhaps the best characterized example of a modifier and target gene in mammals, and similar studies are likely to follow. However, this study raises many questions. Does SCNM1 target other RNAs, and if so, which ones? What role does SCNM1 play in the splicing complex? It appears that SCNM1 is a new member of an important class of splicing factors. It is conceivable that variant SCNM1 accounts for other modifier effects or even acts as a quantitative trait locus involved in other complex traits.

With the remarkable success in discovering genes involved in single-gene disorders, attention naturally turns to more complex traits. In contrast to Mendelian traits, genes involved in multigenic traits are notoriously difficult to identify (*12*). Modifier genes are the obvious points of transition from the simple to the complex genetic world. Although phenotype modifiers are probably genetically complex, involving a substantial number of genes, some are amenable to positional cloning efforts, as the Buchner study demonstrates. And, because they modify the phenotypic expression of a known gene, clues are immediately available about the nature of the pathways, networks, and systems that control biological homeostasis.





Genetic networking. (Top) The *med*^{J} allele of the *Scn8a* gene has a mutation that truncates exon 3 and leads to alternative transcripts, one that includes all four exons and another that skips exons 2 and 3 to give much shorter mRNA. The *Scnm1* gene has two variants, one found in C3H and many other inbred strains and another that is found in C57BL/6J and closely related inbred strains. All seven exons are included in the mRNA in the C3H variant, whereas the B6 variant affects splicing so that alternative transcripts are produced, one that lacks exon 7 and another that skips exon 6. **(Bottom)** On the C3H background 10% of the *Scn8a* transcripts are the normal size, whereas this number falls to 5% on the B6 background. This modest difference in the abundance of the normal *Scn8a* transcript determines the severity of the phenotype.

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